Tampere University

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Immunomodulatory Properties of Wood Biochemicals

Effects on Inflammatory Gene Expression and Inflammatory Responses In Vivo

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Effects on Inflammatory Gene Expression and Inflammatory Responses In Vivo

ACADEMIC DISSERTATION
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Tampere University, Faculty of Medicine and Health Technology
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Contents

List of Original Communications

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I Laavola M, Nieminen R, Leppänen T, Eckerman C, Holmbom B and Moilanen E (2015): Pinosylvin and monomethylpinosylvin, constituents of an extract from the knot of *Pinus sylvestris*, reduce inflammatory gene expression and inflammatory responses *in vivo*. J Agric Food Chem. 63(13):3445-53.

II Laavola M, Leppänen T, Eräsalo H, Hämäläinen M, Nieminen R and Moilanen E (2017): Anti-inflammatory Effects of Nortrachelogenin in Murine J774 Macrophages and in Carrageenan-Induced Paw Edema Model in the Mouse. Planta Med. 83(6):519-26.

III Laavola M, Haavikko R, Hämäläinen M, Leppänen T, Nieminen R, Alakurtti S, Moreira VM, Yli-Kauhaluoma J and Moilanen E (2016): Betulin Derivatives Effectively Suppress Inflammation *in Vitro* and *in Vivo*. J Nat Prod. 79(2):274-80.

IV Laavola M, Leppänen T, Hämäläinen M, Vuolteenaho K, Moilanen T, Nieminen R and Moilanen E (2018): IL-6 in osteoarthritis: effects of pine stilbenoids. Molecules. 24(1) doi: 10.3390/molecules24010109.

In addition, some unpublished data are presented.

Abbreviations

ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
Akt/PKB	Protein kinase B
AMPK	Adenosine monophosphate-activated protein kinase
CCR	CC chemokine receptor
COX	Cyclooxygenase
CRP	C-reactive protein
DMARD	Disease-modifying antirheumatic drug
DMBA	7,12-dimethylbenz[a]anthracene
DMEM	Dulbecco´s Modified Eagle´s Medium
DMOAD	Disease-modifying osteoarthritic drug
DMSO	Dimethylsulfoxide
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EP1	Prostaglandin E_2 receptor 1
GC	Glucocorticoid
GR	Glucocorticoid receptor
HIV	Human immunodeficiency virus
HO-1	Hemeoxygenase-1
ICAM-1	Intercellular adhesion molecule 1
IFN-γ	Interferon gamma
IL	Interleukin
IRF	Interferon regulatory factor
IQR	Interquartile range
JAK	Janus kinase
KC	Keratinocyte-derived chemokine
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein
miRNA	MicroRNA
mRNA	Messenger ribonucleic acid
MMP	Matrix metalloproteinase

NF-ϰB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NOS	Nitric oxide synthase
iNOS	Inducible nitric oxide synthase
NSAID	Non-steroidal anti-inflammatory drug
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PG	Prostaglandin
PGE_2	Prostaglandin E_2
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B (Akt)
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SIRT	Sirtuin
SOR	Strength of recommendation
STAT	Signal transducer and activator of transcription
TGF- β	Transforming growth factor beta
TH	Helper T-cell
TIMP	Tissue inhibitors of metalloproteinases
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
Treg	Regulatory T cell
TRPA	Transient receptor potential ankyrin
TRPV	Transient receptor potential vanilloid
Tx	Thromboxane
VCAM-1	Vascular cell adhesion protein 1
XTT	2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide

Abstract

The inflammatory response is usually beneficial but when it is inappropriately directed, dysregulated or prolonged it may cause tissue injury or trigger the development of inflammatory diseases like osteoarthritis (OA), rheumatoid arthritis (RA) or asthma. At present, the treatment of inflammatory diseases is far from satisfactory. The symptoms might be relieved but the disease cannot necessarily be cured and furthermore the existing therapies have adverse effects.

Inflammation stimulates the activation of inflammatory cells and this enhances the expression of an array of inflammatory genes: cytokines, chemokines, enzymes, adhesion molecules and other factors involved in inflammation. Bacterial products and pro-inflammatory cytokines induce the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), the former enzyme catalyzing the synthesis of nitric oxide (NO) and the latter producing prostaglandin E_2 (PGE_2). These factors together with proinflammatory cytokines such as interleukin-6 (IL-6) and chemokines like monocyte chemoattractant protein 1 (MCP-1) are important mediators and thus possible anti-inflammatory drug targets in inflammatory diseases.

Trees are rich in polyphenolic compounds such as terpenoids; these compounds might be a potential source of immunomodulatory molecules. The pharmacological properties of wood biochemicals are not well known but in traditional medicine, various extracts and products including extracts of Scots pine have been successfully used as anti-inflammatory treatments. A landmark success story in the history of drug discovery is the development of acetylsalicylic acid (Aspirin®) from salicin which is present in extracts of willow bark.

In the present study, pinosylvin and monomethyl pinosylvin, stilbenoid constituents of pine knot extract, were found to possess anti-inflammatory properties *in vitro* in cell models and *in vivo* in the mouse, possibly acting through several mechanisms i.e. the upregulation of HO-1 (hemeoxygenase-1) levels, inhibition of NF-κB mediated transcription and suppression of the expression of inflammatory genes, particularly iNOS, IL-6 and MCP-1. In addition, pinosylvin and monomethyl pinosylvin exerted beneficial effects on cartilage homeostasis by inhibiting IL-6 production and by increasing aggrecan expression in primary human OA chondrocytes.

Nortrachelogenin, a lignan compound present in pine knot extract, reduced the acute inflammatory response in mice and inhibited the production of several inflammatory factors such as NO, PGE_2, IL-6 and MCP-1 in macrophages *in vitro*. A proteasome inhibitor, lactacystin, reversed the effect of nortrachelogenin on iNOS expression pointing to a post-transcriptional mechanism of action.

The anti-inflammatory properties of betulin, betulinic acid and 16 semisynthetic betulin derivatives were also investigated. Betulin is found in substantial amounts in birch bark. Three betulin derivatives selectively inhibited the expression of iNOS in a post-transcriptional manner. Interestingly, a novel pyrazolobetulinic acid derivative was discovered, which suppressed the expression of IL-6, MCP-1 and COX-2 in addition to iNOS. The *in vitro* anti-inflammatory effect was also observed in *in vivo* experiments.

The results revealed completely new anti-inflammatory properties of wood derived biochemicals, which could be utilized in the development of novel anti-inflammatory treatments and at the same time increase the value of the side products of the pulp and paper industry.

Tiivistelmä

Tulehdusvaste on yleensä elimistölle hyödyllinen, mutta pitkittyessään tai kohdistuessaan väärin se voi aiheuttaa kudosvaurion ja/tai johtaa tulehdussairauksien kuten nivelrikon, nivelreuman tai astman kehittymiseen. Nykyiset tulehdussairauksien hoidot eivät ole riittäviä. Niillä voidaan lievittää sairauden oireita, mutta ne eivät paranna sairautta. Lisäksi nykyisillä tulehduslääkkeillä on usein haittavaikutuksia, jotka rajoittavat niiden käyttöä. Tehokkaiden tulehduslääkkeiden kehityksessä tavoitteena on löytää molekyylejä, jotka rauhoittavat tulehdusta mutta eivät häiritse immuunipuolustusjärjestelmän normaalia toimintaa.

Tulehdus saa tulehdussolut käynnistämään tulehdusgeenien aktivoitumisen ja sytokiinien, kemokiinien, entsyymien, adheesiomolekyylien sekä muiden tulehdustekijöiden vapautumisen. Bakteerit ja tulehdusta voimistavat sytokiinit lisäävät indusoituvan typpioksidisyntaasin (iNOS:n) ja syklo-oksigenaasin (COX-2:n) ilmentymistä, jotka katalysoivat typpioksidin (NO) ja prostaglandiini E_2:n tuottoa. Nämä tulehdustekijät yhdessä tulehdusta voimistavien sytokiinien kuten interleukiini-6:n (IL-6) ja kemokiinien kuten monosyyttien kemoattraktanttiproteiini-1:n (MCP-1) kanssa, välittävät tulehduksen etenemistä ja ovat mahdollisia tulehdusta estävien lääkkeiden vaikutuskohteita tulehdussairauksissa.

Puut sisältävät lukuisia polyfenolisia yhdisteitä ja terpenoideja, joten ne voisivat olla mahdollinen lähde etsittäessä tulehdusta estäviä yhdisteitä. Puusta peräisin olevien biokemikaalien farmakologiset vaikutukset tunnetaan huonosti. Perinteisessä lääketieteessä mm. männyn uutteita on käytetty tulehdussairauksien hoidossa. Lääkekehityshistoria tuntee myös aiemman menestystarinan puusta peräisin olevasta salisiinista kehitetystä lääkeaineesta, asetyylisalisyylihaposta eli Aspirinista®.

Tässä tutkimuksessa männyn oksauutteesta peräisin olevilla stilbenoideilla pinosylviinillä ja monometyylipinosylviinillä havaittiin olevan tulehdusta estäviä vaikutuksia *in vitro* solumalleissa ja *in vivo* hiirissä. Vaikutukset perustuivat mahdollisesti hemioksygenaasi-1:n (HO-1) lisääntymisestä aiheutuneen transkriptiotekijä NF-κB:n aktivaation vähentymiseen. Sen seurauksena tulehdusgeenien iNOS, IL-6 ja MCP-1:n ilmentyminen laski. Lisäksi yhdisteillä oli hyödyllinen vaikutus ihmisen rustossa vallitsevaan anabolisten ja tulehdusta

voimistavien / katabolisten yhdisteiden tasapainoon. Yhdisteet estivät IL-6:n tuottoa sekä lisäsivät aggrekaanin ilmentymistä ihmisen primäärirustosoluissa.

Nortrakelogeniini, männyn oksauutteesta eristetty lignaani, vähensi akuuttia tuledusreaktiota hiirimallissa ja esti tulehdustekijöiden NO, PGE_2, IL-6 ja MCP-1 tuottoa aktivoiduissa makrofageissa. iNOS-vaikutus voitiin kumota käyttäen proteosomi-inhibiittori laktakystiiniä, mikä viittaa posttranskriptionaaliseen säätelyyn.

Tutkimme myös betuliinin, betuliinihapon ja 16 puolisynteettisen betuliinijohdannaisen tulehdusta estäviä vaikutuksia. Betuliinia voidaan eristää merkittäviä määriä koivun kuoresta. Kolme betuliinijohdannaista esti selektiivisesti iNOS:n ilmentymistä posttranskriptionaalisesti ja pyratsolobetuliinihappo esti iNOS:n ekspression lisäksi IL-6:n, MCP-1:n ja COX-2:n ilmentymistä. Pyratsolobetuliinihapolla oli *in vitro* vaikutusten lisäksi tehoa myös *in vivo*.

Tulokset toivat ilmi puusta peräisin olevien yhdisteiden aiemmin tuntemattomia tulehdusta estäviä vaikutuksia, joita voitaisiin hyödyntää uusien tulehduslääkkeiden kehityksessä ja samanaikaisesti saada lisäarvoa metsäteollisuuden hyödyntämättömille sivuvirroille.

Introduction

In the 1970s, beneficial effects of xylitol for dental health were discovered, and in the 1990s, plant sterols were found to reduce cholesterol in humans. Both of those are good examples of adding value to the side products of wood industry. Xylitol can be produced from birch and sterols from pine. Wood derived extracts and compounds have had an important role in the history of drug discovery. The most famous success story is the development of acetylsalicylic acid (Aspirin®) from salicin found in the bark of the willow tree to becoming the world's number one pain killer. There is still undiscovered potential in wood biochemicals to be utilized in drug research. Nonetheless, the pharmacological properties of wood derived compounds are not known very well.

The number of new drugs reaching the market has been declining for several years. The proportion of new entities derived from either synthetic chemistry or biological therapies has increased at the expense of small molecule drugs. Nonetheless, still about every second new small molecule drug has its origins in nature. The current situation has led to a reassessment of the potential of nature derived compounds by the pharmaceutical industry. From a historical perspective, natural products have played an important role in the discovery and development of anti-inflammatory drugs. In addition to acetylsalicylic acid, another example is the immunosuppressant drug cyclosporine originating from the fungus *Tolypocladium inflatum* which revolutionized the prognosis of organ transplant patients since its introduction in the 1980s, by preventing transplant rejection.

The treatment of inflammatory diseases is challenging because one needs to strike a balance between defensive immune responses and the suppressive effect on an aberrant inflammatory response driving the disease. Unfavorable adverse effects of anti-inflammatory drugs have increased the interest in identifying highly specific and targeted agents. Unfortunately, many candidates which appeared promising in the pre-clinical phase have failed to demonstrate a therapeutic effect in the clinical phase, for instance, because of the compensatory pathways developing in the human body. Currently, the treatment of many inflammatory diseases is unsatisfactory. The symptoms may be relieved but the disease cannot be cured, for example, this is the situation in osteoarthritis. The development of novel biological drugs has improved

the treatment of rheumatoid arthritis but these agents are not effective in all patients and their safety profile is sometimes problematic.

The high prevalence of inflammatory diseases in conjunction with the limited efficacy and frequent adverse effects related to existing drug therapies underline the urgent need to develop new anti-inflammatory drugs. Compounds derived from wood and related modified molecules which possessed the capability to modulate the expression of inflammatory genes, could revolutionize the treatment of many common inflammatory diseases such as osteoarthritis, rheumatoid arthritis, asthma and allergies.

The aim of the present study was to identify and modify wood biochemicals and to investigate if these molecules could modulate the expression of inflammatory genes and regulate the cartilage homeostasis and subsequently to study their role in the inflammatory response.

Review of the literature

1 Inflammation

The immune system recognizes pathogens, irritants and cell injury; in response, it induces protective reactions, which are crucial for the survival of the host. These defensive immune responses are called inflammation. The classical signs of inflammation are redness, swelling, heat, pain and loss of function. Inflammation can be classified as acute or chronic. Acute inflammation is the initial response to tissue injury, it is typically rapid in onset and of short duration. Acute inflammation can be characterized by the increased movement of plasma and leukocytes (particularly neutrophils and monocyte/macrophages) from the bloodstream into the inflamed tissues. Chronic inflammation is a response of prolonged duration, in which active inflammation, tissue injury and healing all are present at the same time. Chronic inflammation is characterized by infiltration of mononuclear cells i.e. monocytes/macrophages and lymphocytes as well as the activation of repair mechanisms like fibrosis and angiogenesis. Chronic unresolved inflammation is also associated with an increased risk of several common diseases like arthritis, cancer or diabetes. (Crusz & Balkwill 2015, Robbins *et al.* 2015)

Immune response can be divided into innate and adaptive immunity. Innate immunity is non-specific in its nature and provides the early line defence against microbes. The main components of innate immunity are:

- physical (skin and epithelia) and chemical (reduced pH, secretion of chemicals and enzymes) barriers
- inflammatory cells mainly neutrophils, macrophages, dendritic cells and natural killer cells
- complement and kinin systems
- inflammatory mediators including cytokines, chemokines, eicosanoids and reactive oxygen and nitrogen species

In contrast to innate immunity, adaptive immune system specifically targets the inducing agent and takes days rather than hours to develop. Adaptive immunity is separated into humoral immunity and cell-mediated immunity. Humoral immunity is mediated by the antibodies secreted by B-cells. Antibodies recognise antigens and bind to extracellular microbes thus blocking their ability to infect the host cell. In addition, antibodies promote the infected cells to be destroyed by phagocytes and trigger the release of inflammatory mediators. Cell-mediated immunity is mediated by T-cells and is targeted to the intracellular microbes that can survive and proliferate in host tissue cells or in phagocytes. In cell-mediated immunity, cytotoxic T-lymphocytes induce apoptosis in host cells infected with microbes and also in tumour cells; while helper T-lymphocytes activate macrophages and natural killer cells, enabling them to destroy pathogens and stimulate cells to secrete a variety of cytokines. Different types of subsets of helper T-cells Th1, Th2, Th9, Th17, Th22, regulatory T cells (Treg) and the signature cytokines secreted by the subsets are presented in Figure 1. (Eyerich *et al.* 2009, Raphael *et al.* 2015)

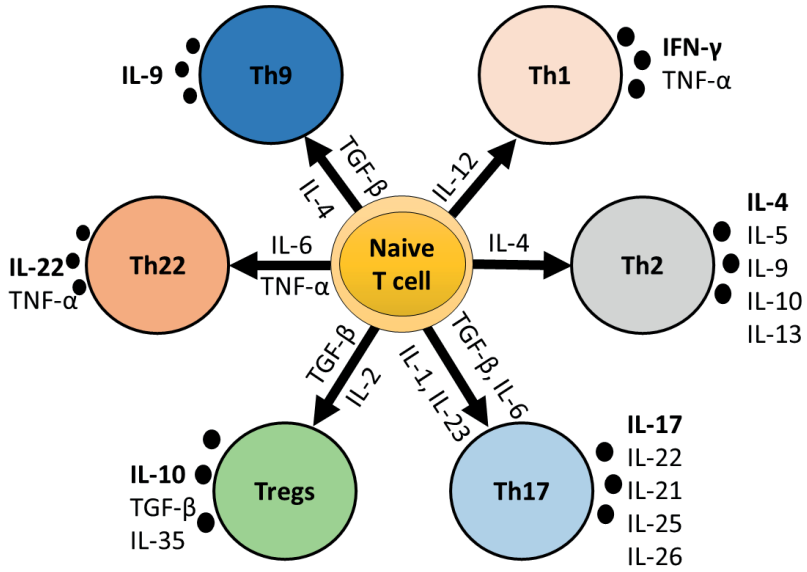


Figure 1. Naive Th cells can be polarized into different T cell subtypes Th1, Th2, Th9, Th17, Th22 and regulatory T (TReg) cells depending on the cytokine environment. In the presence of IL-12, T cells differentiate into Th1 and in the IL-4 environment into Th2 cells; when IL-6 and TGF-β are present, naive T cells can differentiate into Th17 cells. The production of Treg cells is promoted by TGF-β and IL-2. In the presence of TGF-β, T cells differentiate into Th9 cells. Th22 cells are separated from Th17 cells in that they can secrete IL-22 and TNF-α but not IL-17. The signature cytokines secreted by different subtype of T cells are shown in bold. Modified from (Raphael *et al.* 2015).

Macrophages are key cells in innate and adaptive immunity. Under normal physiological conditions, a small number of macrophages are located in tissues where they maintain homeostasis, removing apoptotic cells and remodeling tissues. These tissue macrophages are established during embryonic development. Most of the macrophages that accumulate at diseased sites have typically infiltrated from blood circulating monocytes and differentiated into macrophages in the inflamed tissues. These cells are multipurpose cells - phagocytizing infected cells or presenting antigens to lymphocytes and mediating inflammatory response between innate and adaptive immunity. Macrophages either promote or restrain inflammation by secreting various cytokines and other soluble factors. (Robbins *et al.* 2015)

Currently it is accepted that there are different macrophage phenotypes; these are categorized generally as classically activated (M1) and alternatively activated (M2) macrophages, and the latter is further divided into various subtypes. Classical activation is induced during inflammation and generally promotes inflammatory processes whereas alternatively activated macrophages participate in the resolution of inflammation and tissue healing. The focus of this thesis work is on classically activated macrophages. We used lipopolysaccharide (LPS) to activate the classical transcriptional cascade initiated by the Toll-like receptor 4 (TLR4) receptor. TLR4 co-operates with proteins: CD14, LPS binding protein and MD-2 and signaling cascade further activates NF-κB and MAPK pathways. (Hume 2015, Tan & Kagan 2014)

The immune response is usually beneficial but when it is inappropriately directed or inadequately controlled it becomes a critical contributor to the pathophysiological processes inherent in several inflammatory diseases like arthritis.

1.1 Treatment of inflammation

Over the past decade, it has become widely accepted that inflammation is a driving force in many chronic diseases, including arthritis, asthma, allergy, cancer, diabetes, Alzheimer's disease, atherosclerosis and obesity. An optimal anti-inflammatory drug should suppress the inappropriate inflammatory response without interfering with normal homeostasis.

The drugs currently used to treat inflammation, particularly arthritis, which was the focus of the current thesis, can be divided into three groups:

- non-steroidal anti-inflammatory drugs (NSAIDs)
- glucocorticoids
- disease-modifying antirheumatic drugs (DMARDs)

NSAIDs were discovered more than 100 years ago and they are the most commonly used medicines all around the world. On the other hand, NSAIDs are associated with safety and tolerability concerns, which should be considered when prescribing NSAIDs to patients at risk. NSAIDs inhibit prostanoid biosynthesis through their activity on the two cyclo-ogygenase enzymes COX-1 and COX-2 and thereby reduce the synthesis of prostaglandins, prostacyclin and thromboxane. Inhibition of COX-1 is associated with gastrointestinal adverse effects. This knowledge led to the development of COX-2 selective inhibitors, the so-called coxibs. Evidence of increased risk of myocardial infarction and other thrombotic events resulted in the withdrawal of rofecoxib, a selective COX-2 inhibitor, from the market. Nowadays, it is understood that it is not COX-2 selectivity in itself but merely the degree to which COX-2 is inhibited that increases the risk of thromboembolic adverse effects in patients with cardiovascular diseases. Widely used NSAIDS and their COX selectivity is listed in Table 1. Novel compounds with improved safety profile are under development e.g. nitric oxide releasing NSAIDs and compounds with improved pharmacokinetics. (Altman *et al.* 2015, Brune & Hinz 2004, Brune & Patrignani 2015)

Table 1. Non-steroidal anti-inflammatory drugs and their cyclo-oxygenase selectivity modified from (Brune & Patrignani 2015, Warner & Mitchell 2004)

COX isoform selectivity	Drug
COX-2 selective	Celecoxib
	Etoricoxib
	Lumiracoxib*
	Parecoxib (prodrug, metabolized to valdecoxib *in vivo*)
	Rofecoxib**
	Valdecoxib**
COX-2 preferred	Etodolac
	Meloxicam
	Nabumetone
	Nimesulide*

COX isoform selectivity	Drug
Non-selective	Aceclofenac
	Acetylsalicylic acid
	Diclofenac
	Diflunisal
	Fenoprofren
	Flurbiprofen
	Ibuprofen
	Indomethacin
	Ketoprofen
	Ketorolac
	Lornoxicam
	Naproxen
	Niflumic
	Piroxicam
	Sulindac
	Tolfenamic acid
	Zomepirac

*withdrawn from the market because of liver toxicity
**withdrawn from the market because of cardiovascular events

Glucocorticoids (GCs) are the most powerful of the currently known anti-inflammatory drugs. They are endogenous stress hormones that act via glucocorticoid receptors (GRs), regulating many physiological functions such as glucose metabolism and metabolic homeostasis, cell proliferation, development, stress responses, cognition and mental health, as well as inflammation. Therapeutically, synthetic GCs are used to treat various inflammatory conditions including asthma and allergy, rheumatoid arthritis and other autoimmune diseases as well as preventing transplant rejection. There are many widely used orally administered synthetic GCs, e.g. prednisone, prednisolone and dexamethasone. There are also two GCs normally administered intra-articularly, methylprednisolone and triamsinolone, while budesonide, beclomethasone, fluticasone and the pro-drug ciclesonide which are inhaled into the lungs. Unfortunately, high doses and long term GC treatments increase the risk of severe adverse effects, e.g. skin and muscle atrophy, impaired wound healing, increased risk of infections, moon face, glaucoma,

hyperglycemia and diabetes, osteoporosis, cardiovascular complications and behavioral changes. (Adcock & Mumby 2017, Vandewalle *et al.* 2018)

GCs have multiple anti-inflammatory effects through activating anti-inflammatory genes and suppressing pro-inflammatory genes. Most of the effects of GCs are mediated through genomic pathways but non-genomic effects have also been described. GCs bind to cytoplasmic glucocorticoid receptors which then translocate to the nucleus. In the nucleus, the GC-GR complex binds to glucocorticoid response elements (GREs) present in the promoter region of several genes or to co-activator molecules, which both lead to activation of genes encoding anti-inflammatory proteins such as mitogen-activated kinase phosphatase-1 (MKP-1), inhibitor of nuclear ϰB (IϰB) or secretory leukoprotease inhibitor. In addition, many metabolic effects of CGs are mediated through this so-called transactivation mechanism. GCs can also down-regulate the activity of inflammatory transcription factors. For example, activator protein 1 (AP-1) binds to GR and prevents its interaction to GRE and this also blocks the effects of AP-1. NF-kB is another inflammatory transcription factor inhibited by GCs. These mechanisms lead to suppressed transcription of various pro-inflammatory cytokines and other factors. In the non-genomic pathway, GR modulates the activity of kinases including PI3K and AKT. Post-transcriptional effects have also been reported. GCs are able to target tristetraprolin (TTP) and Hu antigen R (HuR) and several microRNAs (miRNAs), which control mRNA decay and stability. (Barnes 2011, Clayton *et al.* 2018, Panettieri *et al.* 2019, Vandewalle *et al.* 2018)

DMARDs are an important group of drugs because they influence also the progress of the inflammatory disease e.g. joint destruction in arthritis (Table 2). Conventional DMARDs are a heterogenic group of chemical compounds including methotrexate, sulfasalazine, (hydroxyl)chloroquine, leflunomide, penicillamine and gold compounds. Immunosuppressants such as calcineurin inhibitors, are used in the treatment of autoimmune diseases and in the prevention of transplant rejection. Limited efficacy connected to multiple adverse effects associated with the conventional therapies and increased understanding of immune system stimulated the search for targeted therapies in the 1990s. The first biological TNF-α inhibitors infliximab and etanercept were approved in 1998 in US. (Laev & Salakhutdinov 2015, Wiseman 2015)

More recently, the first targeted synthetic small molecular DMARDs, Janus kinase inhibitors and phosphodiesterase inhibitors, have entered the market (Caporali & Zavaglia 2018).

Table 2. Disease modifying anti-rheumatic drugs. Data collected from (Bondeson 1997, Laev & Salakhutdinov 2015, Mohamed *et al.* 2016, Rang *et al.* 2016, Richez *et al.* 2017, Wiseman 2015)

Type	Drug	Mechanism of action
Conventional synthetic DMARDSs	Methotrexate	Inhibits folate-dependent enzymes leading to an impaired function of lymphocytes. Inhibition of NF-κB activation and increased IL-10 production.
	Sulfasalazine	Precise mechanism of action is not known; breaks down to sulfapyridine and 5-aminosalicylic acid by gut bacteria, increases the release of the anti-inflammatory factor adenosine, alters gut microbiome, inhibits lymphocyte activity, decreases the production of pro-inflammatory cytokines.
	Chloroquine and hydroxychloroquine	Precise mechanism of action is not known; Inhibits lymphocyte activity, decreases the production of pro-inflammatory cytokines and scavenger of ROS.
	Leflunomide	Prodrug, converted to the active metabolite teriflunomide *in vivo*; inhibits the enzyme dihydro-orotate dehydrogenase leading to reduced formation of pyrimidine nucleotides and suppression of lymphocyte proliferation, inhibits the production of pro-inflammatory cytokines and ROS.
	Calcineurin inhibitors cyclosporine and tacrolimus	Inhibit the phosphatase calcineurin leading to the inhibition of the activation of the transcription factor NF-AT, which is critical for T-lymphocyte proliferation and cytokine production, primarily targets T-lymphocytes but as a consequence, suppresses many other aspects of the inflammatory reaction.
	Aurothiomalate	Precise mechanism of action is not known; Increases in MKP-1 expression, accumulates in macrophages, decreases the production of pro-inflammatory cytokines and MMP enzymes.
	Penicillamine	Precise mechanism of action is not known; chelates metal ions, inactivates ROS, inhibits lymphocyte activity, decreases the production of pro-inflammatory cytokines.
Targeted synthetic DMARDs	Tofacitinib, baricitinib and upadacitinib	Janus kinase (JAK) inhibitors, inhibit leukocyte activation and the production of pro-inflammatory cytokines.
	Apremilast	Phosphodiesterase inhibitor, increases intracellular cAMP levels, inhibits leukocyte activation and the production of pro-inflammatory cytokines.

Type	Drug	Mechanism of action
Biological DMARDs	Adalimumab, certolizumab, etanercept, golimumab and infliximab	TNF inhibitor, inhibits the biological activity of the pro-inflammatory cytokine TNF.
	Anakinra	IL-1 inhibitor, recombinant human IL-1 receptor antagonist, inhibits the biological activity of the pro-inflammatory cytokine IL-1.
	Tocilizumab	IL-6 inhibitor, human monoclonal antibody against IL-6 receptor, inhibits the biological activity of the pro-inflammatory cytokine IL-6.
	Rituximab	B-cell directed therapy, a chimeric monoclonal antibody against the protein CD20 which is expressed on mature B-lymphocytes, triggers cell death in CD20 positive B-cells.
	Abatacept	T-cell directed therapy, humanized fusion protein in which the extracellular domain of human cytotoxic T lymphocyte-associated antigen 4 (CTL4) is combined to human immunoglobulin, inhibits CD28 – CD80/86 mediated co-stimulatory signal in T-lymphocyte activation leading to reduction in T-lymphocyte proliferation and cytokine production.

Although the biological treatments have been described as a success story in the treatment of inflammatory diseases, they are not effective in all patients and the therapies are not without problems (Dalal *et al.* 2018). Some patients will develop anti-drug antibodies which lead to reduced clinical response and may increase the risk of adverse effects (Schaeverbeke *et al.* 2015). The high prevalence of inflammatory diseases and the insufficient clinical presentation of the existing therapies are the driving forces to develop new anti-inflammatory drugs.

2 Inflammatory mediators

The soluble chemicals released from activated, injured or dying cells can be considered as mediators of inflammation. Their role is to co-ordinate the development of the inflammatory response. Inflammatory mediators include:

- vasoactive amines
  - histamine
  - serotonin
- plasma endopeptidases
  - the kinin system that produces bradykinin
  - the complement system that produces proteins that interact with various components of the inflammatory response
  - the clotting system that increases vascular permeability and chemotactic activity of the leukocytes
- eicosanoids and other lipid mediators
  - prostaglandins and other prostanoids
  - leukotrienes
  - lipoxins, resolvins, protectins and maresins
  - platelet activating factor
- cytokines and chemokines
- nitric oxide and other reactive nitrogen species (RNS)
- reactive oxygen species (ROS)

Inflammatory mediators activate, regulate and terminate inflammation. Regulation of the synthesis or activity of the inflammatory mediators is one of the major approaches adopted in the anti-inflammatory drug development. The focus in the present PhD thesis has been on nitric oxide, cytokine IL-6, chemokine MCP-1 and prostaglandin PGE_2 which are reviewed in more detail in the next sections.

2.1 Nitric oxide

Nitric oxide (NO) is a gaseous signaling molecule that is synthesized from amino acid L-arginine in a reaction catalyzed by nitric oxide synthase (NOS) (Figure 2)

(Predonzani *et al.* 2015). Three isoforms of NOS have been characterized; two constitutive NOS isoforms were originally detected in endothelial cells (eNOS) and in neurons (nNOS) and an inducible isoform iNOS in macrophages (Blottner & Baumgarten 1992, Lyons *et al.* 1992, Palmer & Moncada 1989).

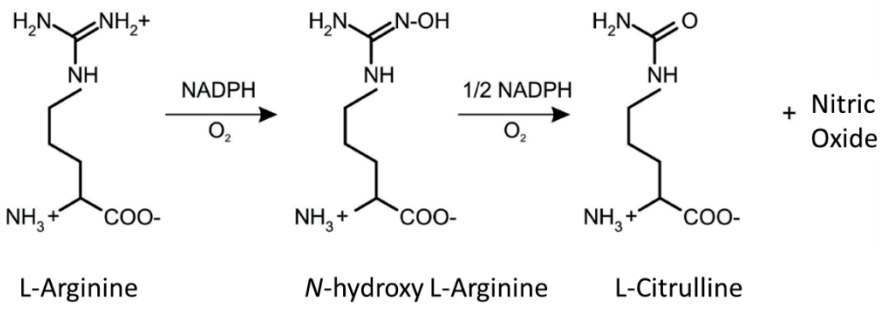


Figure 2. Synthesis of nitric oxide from L-arginine catalysed by NOS

In 1980, Robert F. Furchgott identified an endothelium-derived relaxing factor (EDRF) that evoked the relaxation of the endothelial muscle cells (Furchgott & Zawadzki 1980). Later, two research groups Ignarro et al. and Moncada´s research group proved that this factor was nitric oxide (Ignarro *et al.* 1987, Palmer *et al.* 1987). NO is involved in many physiological and pathophysiological processes e.g. it modulates blood flow, neural activity and immune defence mechanism (Mobasheri 2013, Predonzani *et al.* 2015).

The first signs of the role of NO in inflammation dates from 1985 when Stuehr and Marletta observed that lipopolysaccharide (LPS) activated mouse macrophages produced significant amounts of nitrite and nitrate (Stuehr & Marletta 1985) which was later understood to reflect the synthesis of NO which is rapidly metabolized to nitrite and nitrate in cell culture conditions. The production of NO has also been demonstrated in other cells. The effects of NO in inflammation and immunity are related to the environment where NO is produced as well as to the amount of NO being produced. High levels of NO mediate proinflammatory and tissue-destructive effects. On the other hand, NO exerts also regulatory and protective effects in some inflammatory responses, and NO-derived radicals form one of the endogenous antimicrobial defense systems (Predonzani *et al.* 2015). The role of nitric oxide in different immune cells is presented in Table 3.

Table 3. Nitric oxide in immune cells (modified from Predonzani et al. 2015)

Type of immunity	Cell	Features	References
Innate immunity	Macrophage		
	M1	High level NO production Expression of iNOS NO-mediated cytotoxicity	(Mills *et al.* 2000)
	M2	Low level NO production Immune suppression	
	Natural killer cells	NO-mediated cytotoxicity NO-mediated cell fitness	(Furuke *et al.* 1999)
	Mast cells	NOS expression NO-mediated cell adhesion and function	(Gilchrist *et al.* 2004)
	Myeloid-derived suppressor cells	iNOS expression	(Angulo *et al.* 2000)
	Dendritic cells	NOS expression Pathogen clearance	(Lu *et al.* 1996)
Adaptive immunity	Lymphocyte		
	T-cell	T-cell differentiation	(Niedbala *et al.* 2002)
	B-cell	Reduced level of NO	(Giordano *et al.* 2014)

Interestingly, increased iNOS expression and NO production in macrophages and other cells are involved in the pathogenesis of various inflammatory diseases such as asthma, colitis, psoriasis, neurodegenerative disorders, cancer and arthritis (Joubert & Malan 2011). The role of NO in OA and RA has been studied intensively after finding increased production of NO in chondrocytes from patients with RA and OA. NO seems to be a proinflammatory and destructive mediator in the cartilage. Excess production of NO in OA leads to the inhibition of type II collagen and proteoglycan synthesis, activation of metalloproteinases and chondrocyte apoptosis. (Vuolteenaho *et al.* 2007).

2.1.1 Inducible nitric oxide synthase (iNOS)

In contrast to nNOS and eNOS which are constitutively expressed in small amounts, for instance, in endothelial cells, platelets and neurons, iNOS is produced in large amounts at sites of injury and inflammation. The activity of the constitutive forms of NOS is mainly dependent on an increase in the intracellular level of calcium but

iNOS has been described as a "calcium-insensitive" enzyme, likely due to its tight non-covalent interaction with calmodulin and Ca^{2+}. (Kobayashi 2010)

The human iNOS gene is located in chromosome 17; it has 26 exons and 25 introns. The iNOS enzyme is a homodimer composed of two 130 kDa subunits consisting of 1153 amino acids. Each subunit includes an oxygenase domain and a reductase domain. It is noteworthy that dimerization of iNOS is required for enzyme activity. If the dimerization mechanism is disrupted, the production of NO via iNOS is suppressed. The oxygenase domain has binding sites for the substrate L-arginine, heme and the redox cofactor tetrahydrobiopterin (BH_4). The oxygenase and reductase domains are separated by a calmodulin binding region. The reductase domain contains binding sites for flavin mononucleotide and flavin-adenine dinucleotide in addition to several consensus sites for the electron donor species. Inhibition of iNOS can be achieved by inhibition of substrate or co-factor finding but also by preventing the dimerization which is necessary to form the biologically active dimeric enzyme.(Alderton *et al.* 2001)

iNOS knockout mice are more susceptible to certain infections than their wild type counterparts, whereas they are resistant to sepsis-induced hypotension which implies that iNOS plays an important role in immune defence. iNOS is expressed *in vitro* by various stimuli including LPS, IFNs, IL-1β and TNF-α.(Huang 2000, Kobayashi 2010)

iNOS-selective and nonselective NOS inhibitors have been investigated in the treatment of several iNOS-mediated diseases. The results in the animal models of sepsis, lung inflammation, arthritis and pain of different iNOS inhibitors (1400W, GW274150, GW273629, AR-C102222, ON01714, L-NIL, SC-51 and dimerization inhibitors BBS-1 and KD7332) have shown promising results. (Bonnefous *et al.* 2009, Joubert & Malan 2011)

A few NOS inhibitors have also been investigated in short-term clinical studies but the results so far have been less promising than those obtained in preclinical studies especially to the rather promising findings *in vitro* and *in vivo* (Barbanti *et al.* 2014, Hellio le Graverand *et al.* 2013, Seymour *et al.* 2012).

2.1.2 Regulation of iNOS expression

iNOS gene has been shown to be regulated extensively by transcriptional mechanisms. There are certain differences in iNOS promoters between species but all mammalian iNOS genes exhibit homologies to binding sites for many

transcription factors i.e. AP-1, C/EBP, CREB, GATA, HIF, IRF-1, NF-AT, NF-κB, NF-IL6, Oct-1, PARP1, PEA3, p53, Sp1, SRF, STAT-1α and YY1. iNOS promoters from all species contain a TATA box in the transcription start site and close to the TATA box, there are binding sites for the transcription factors NF-κB, NF-IL6, octamer factors and for transcription factors induced by TNF-α. (Korhonen *et al.* 2005, Pautz *et al.* 2010)

NF-κB seems to be a significant target and its modulation can lead to either activation or inhibition of iNOS expression. For example, stimulants like LPS, IL-1β and TNF-α have been shown to induce iNOS expression in different cells by activating NF-κB. In addition, inhibition of iNOS expression, for example by glucocorticoids, is at least partly a result of the inhibition of NF-κB activation. NF-κB inhibition can be an outcome from direct capture of NF-κB by protein–protein interactions, blockade of nuclear translocation of NF-κB, inhibition of NF-kB transactivation activity or from induced expression of a specific inhibitor of NF-κB named as I-κB. In addition to NF-κB pathway, JAK-STAT and MAPK pathways are important for the activation of human iNOS expression. (Pautz *et al.* 2010)

The iNOS gene is also regulated post-transcriptionally by regulation of mRNA stability. Previously, several RNA binding proteins i.e. HuR, TTP, PABP, A1, PTB, TIAR and KSRP, have been shown to enhance the stability of iNOS mRNA (Jalonen *et al.* 2006, Jalonen *et al.* 2008, Pautz *et al.* 2010). Interestingly, in addition to transcriptional effects, glucocorticoids have been reported to induce the degradation of the murine iNOS mRNA (Korhonen *et al.* 2002).

MicroRNAs are short non-coding nucleotides that are complementary to 3'-UTR mRNA sequences. miRNAs have been shown to negatively regulate mRNA stability by accelerating its degradation or to exert a translational blockade of protein synthesis. Three miRNAs regulating human iNOS gene have been identified. (Guo & Geller 2014) miR-146a has been shown to indirectly regulate iNOS expression possibly through the TLR4-NF-κB pathway. miR-939 and miR-26a have been reported to suppress the expression of iNOS protein via translational blockade by binding 3'-UTR. (Guo & Geller 2014, Tan *et al.* 2018)

2.2 Interleukin-6

The cytokine interleukin-6 (IL-6) is a glycosylated 184 amino acid protein, which was identified by Hirano et al. in 1986 (Hirano *et al.* 1986). When it was first identified, it was shown to promote the activation of T cells, differentiation of B cells and

regulation of the acute-phase response. IL-6 is a mediator involved in inflammation, immune response and hematopoiesis. It can activate target cells via three different receptor-mediated pathways: classical activation, trans-signaling and the quite recently discovered trans-presentation (Figure 3). In classical activation, IL-6 binds to a specific cell surface receptor (IL-6R), a type I transmembrane receptor of 80 kDa which associates with the membrane bound glycoprotein 130 (gp130) and initiates intracellular signaling. In trans-signaling, IL-6 binds to the soluble form of IL-6R (sIL-6R) and the complex of IL-6 and sIL-6R binds to cell membrane bound gp130, which subsequently initiates intracellular signaling. In the trans-presentation, IL-6 bound to membrane-bound IL-6R displayed on the cell surface, for example on dendritic cells is presented to gp130 molecules expressed on nearby cell types like lymphocytes. IL-6R is mainly present in hepatocytes, neutrophils, monocytes and $CD4^+$ T-cells while gp130 is expressed on all cells. (Heink *et al.* 2017, Hunter & Jones 2015, Jones & Jenkins 2018, Schaper & Rose-John 2015, Tanaka *et al.* 2014)

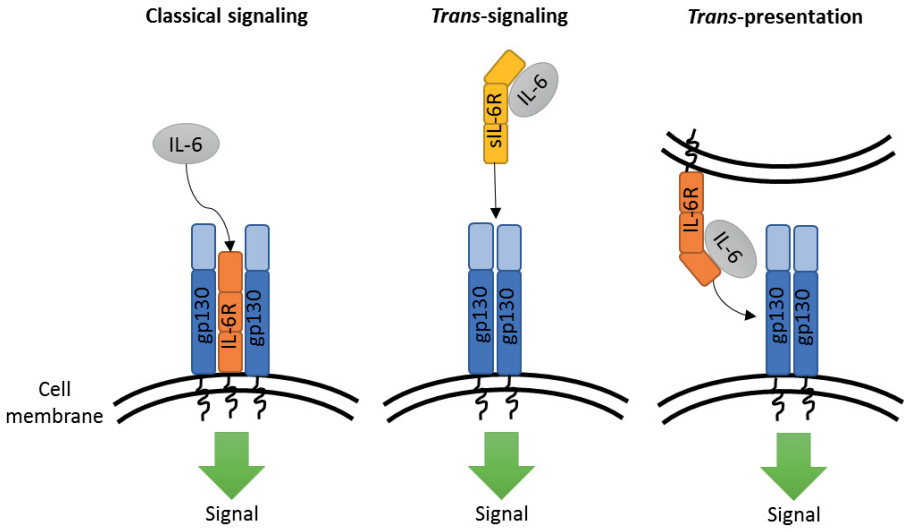


Figure 3. Receptor signaling pathways of IL-6. In classic activation, IL-6 binds to the membrane-bound IL-6R which then associates with cellular membrane bound glycoprotein 130 (gp130) to initiate intracellular signaling. In trans-signaling, IL-6 binds to the soluble form of IL-6R (sIL-6R) and the complex of IL-6 and sIL-6R binds to cellular membrane bound gp130, which initiates intracellular signaling. In trans-presentation, IL-6 bound to membrane-bound IL-6R displayed in the cell surface is presented to gp130 expressed in another cell to activate signaling. Modified from (Jones & Jenkins 2018).

IL-6 can act as a pro-inflammatory or regulatory / anti-inflammatory factor (Table 4). Classic signaling via the membrane bound IL-6R induces acute phase response but has also protective and regenerative effects, while trans-signaling is induces mainly pro-inflammatory actions. Trans-presentation is related to neuroinflammation and dendritic cell activity and T-cell commitment. IL-$6^{-/-}$ and IL-$6R^{-/-}$ mice are both viable and have been shown to develop less severe disease in models of various diseases e.g. RA, systemic lupus erythematosus, systemic sclerosis, inflammatory myopathies, Castleman's disease (a lymphoproliferative disorder), experimental autoimmune uveoretinitis and encephalomyelitis. (Jones & Jenkins 2018, Schaper & Rose-John 2015, Tanaka *et al.* 2014)

Table 4. Responses of IL-6 via IL-6R and sIL-6R. Data collected from (Jones & Jenkins 2018, Schaper & Rose-John 2015)

Responses via membrane bound IL-6R (protective and regenerative)	**Responses via soluble IL-6R (pro-inflammatory)**
Initiates acute phase response	Increase chronic inflammation
Inhibits epithelial cell apoptosis	Stimulates endothelial cells
Increase hepatic and pancreatic regeneration	Stimulates smooth muscle cells
Increase intestinal epithelial cell proliferation	Inhibits T-cell apoptosis
	Inhibits T_{reg} differentiation
	Increase the recruitment of mononuclear cells

2.2.1 Regulation of IL-6 expression

Engagement of TLRs leads to the activation of the NF-kB pathway, which is one of the strongest stimuli for the synthesis of IL-6. Furthermore, IL-6 expression can be stimulated by IL-1β and TNF-α. IL-6 expression is regulated both transcriptionally and post-transcriptionally. (Schaper & Rose-John 2015)

In the human IL-6 gene, binding sites have been found for NF-kB, specificity protein 1 (SP1), nuclear factor IL-6 (NF-IL-6), activator protein 1 (AP-1) and IRF 1. Interestingly, some viral products also enhance the DNA-binding activity of NF-kB and NF-IL-6, resulting in an increase in the transcription of IL-6 mRNA. On the other hand, transcription factors such as aryl hydrocarbon receptor, glucocorticoid receptor, estrogen receptor α, p53, retinoblastoma and PPARα suppress IL-6 expression. There is evidence that some microRNAs directly or indirectly regulate expression of IL-6. miR-155 interacts with NF-IL-6 and miR-146a/b (targeting interleukin-1 receptor-associated kinase 1) and miR-223 (targeting STAT3) indirectly suppress activation of IL-6 gene. The stabilization of IL-6 mRNA is promoted by MAPK p38 and ORF-57 whereas RNA-binding proteins, such as TTP and BRF1 and 2, promote IL-6 mRNA degradation. Post-transcriptionally IL-6 mRNA levels are also reduced by miR-365 and miR-608 through a direct interaction with IL-6 3'UTR. IL-6-dependent activation of the JAK-STAT, MAPK, and the PI3K pathway has been described comprehensively. Until now, mainly JAK/STAT activation has been considered as an intracellular target for interfering in pathological IL-6 signaling. (Schaper & Rose-John 2015, Tanaka *et al.* 2014)

2.2.2 IL-6 inhibitors

Normal physiological concentration of IL-6 in the serum of healthy individuals is in the range of 1-5 pg/ml. The concentration of IL-6, however, increases rapidly in disease settings and can reach μg/ml range in septic shock. Concentrations up to 150 ng/ml in patients with autoimmune diseases such as RA have been reported. Because of the wide range of biological activities and the role of IL-6 in the pathology of several diseases, IL-6 has become an important target in drug development. Tocilizumab, a humanized anti-IL-6R monoclonal antibody was the first to be marketed for the treatment of RA and subsequently sarilumab became available.(Hunter & Jones 2015, Tanaka *et al.* 2014)

Another IL-6 inhibitor, siltuximab, an anti-IL-6 chimeric monoclonal antibody was approved in USA and Europe for the treatment of Castleman's disease, an abnormal overgrowth of cells of the lymph system. Various drugs that block the IL-6 pathway are in the pre-clinical or clinical phase in addition to those already on the market (Table 5). Interestingly, there is a drug, olamkicept, targeting the trans-signaling pathway instead of classic activation of IL-6. Increased risk of bacterial infections and development of metabolic changes have been considered to be a

consequence of global blockade of IL-6 activity with tocilizumab. Possibly these kinds of adverse effects could be avoided when only IL-6 trans-signaling is blocked.(Hunter & Jones 2015)

Table 5. Drugs targeting of IL-6 on the market and undergoing development.

Name	Indication	Status	References
Sirukumab	RA	Phase III*	(Thorne *et al.* 2018)
Clazakizumab	RA Cancer	Phase II Phase II	(Weinblatt *et al.* 2015)
Olokizumab	RA	Phase II	(Genovese *et al.* 2014)
EBI-029	Diabetic macular edema	Pre-clinical	(Hunter & Jones 2015)
VHH6	Drug discovery purposes	Pre-clinical	(Adams *et al.* 2017)
Tocilizumab	RA	On the market	(Ohsugi & Kishimoto 2008)
Sarilumab	RA	On the market	(Raimondo *et al.* 2017)
Siltuximab	Castleman's disease	On the market	(van Rhee *et al.* 2010)
Olamkicept	RA and inflammatory bowel diseases	Phase II	(Hunter & Jones 2015)
NI-1201	Autoimmune and inflammatory diseases	Pre-clinical	(Lacroix *et al.* 2015)
Vobarilizumab	RA	Phase II	(Van Roy *et al.* 2015)

*US Food and Drug Administration rejected approval because of safety issues in 2017 and Janssen-Cilag decided to withdraw the application from the European Medicines Agency

2.3 Monocyte chemoattractant protein-1

Chemoattractant cytokines known as chemokines are released in the early phase of inflammation. These small proteins induce chemotaxis in nearby leukocytes which promotes the movement of these cells towards the source of the chemokine. Chemokines are produced by a variety of cells and their role in cell recruitment is biphasic. First, they act on the leukocytes which are rolling along endothelial cells

close to the site of inflammation. This allows the leukocytes to cross the blood vessel wall. Second, the chemokine directs the migration of the leukocytes towards and into the site of inflammation based on a concentration gradient. Monocyte chemoattractant protein-1 (MCP-1; also known as monocyte chemotactic protein-1 and C-C motif chemokine ligand 2, CCL2) was the third purified chemokine found after platelet factor-4 and IL-8 (Daly & Rollins 2003).

MCP-1 is a central chemokine in the inflammatory response. The MCP-1 gene is located in chromosome 17 and the human protein is composed of 76 amino acids being 13 kDa in size. MCP-1 serves as a chemoattractant for monocytes and macrophages, and plays key roles in many immune processes. MCP-1 mediates its effects through CC chemokine receptor 2 (CCR2) which has two alternatively spliced forms namely CCR2A and CCR2B. CCR2A is the major isoform expressed by vascular smooth muscle cells, whereas monocytes and activated NK cells express predominantly the CCR2B isoform. Other MCP family members, MCP-2, -3, -4 and -5, can also activate the CCR2. (Daly & Rollins 2003, Deshmane *et al.* 2009, Gu *et al.* 2000, Panganiban *et al.* 2014)

MCP-1 gene transcription is regulated by AP-1, NF-κB, MAPK and JNK pathways (Bianconi *et al.* 2018). Involvement of miRNA-based regulation of MCP-1 expression is found in human diseases where this chemokine directs inflammatory cell recruitment (Panganiban *et al.* 2014). The expression of miR-124a was decreased in the synoviocytes obtained from rheumatoid arthritis patients. The analyses revealed that MCP-1 contains a putative miR-124a binding site and the overexpression of miR-124a in synoviocytes led to decreased MCP-1 synthesis. This finding was further confirmed using a luciferase reporter construct containing the MCP-1 3' UTR, which showed a decrease in luciferase activity in the presence of miR-124a overexpression (Nakamachi *et al.* 2009). It has also been shown that miR-126 directly binds to MCP-1 resulting in suppressed MCP-1 expression (Panganiban *et al.* 2014).

MCP-1 has been shown to play roles in inflammatory bowel disease, rheumatoid arthritis, cardiovascular diseases, cancer, Alzheimer's disease, neurodegeneration, neuroinflammation and atherosclerosis as well as in asthma (Deshmane *et al.* 2009, Lin *et al.* 2014). In addition, MCP-1$^{-/-}$ mice were found to have an increased bone mass and also augmented amounts of trabecular bone, indicating that MCP-1 acts as a modulator of bone remodeling (Sul *et al.* 2012). Because a number of studies have indicated that MCP-1 plays a critical role in the development of many inflammatory diseases, MCP-1 is considered as a target for the drug treatments (Takahashi *et al.* 2009, Yadav *et al.* 2010). Promising preclinical results in cancer models led to clinical

trials where the MCP-1 antibody, carlumab, has been studied in patients with pulmonary fibrosis or cancer without significant effects. (Lim *et al.* 2016, Raghu *et al.* 2015).

2.4 Prostanoids

Eicosanoids are biologically active lipid mediators derived from arachidonic acid and related fatty acids. They form a complex network in the body that controls many physiological and pathophysiological processes like vascular homeostasis, kidney perfusion, platelet aggregation, gastrointestinal integrity, inflammation and cancer. The main classes of eicosanoids are prostanoids (prostaglandins, prostacyclins and thromboxanes), leukotrienes and lipoxins. The formation of prostanoids is presented in Figure 4. (Korotkova & Jakobsson 2014)

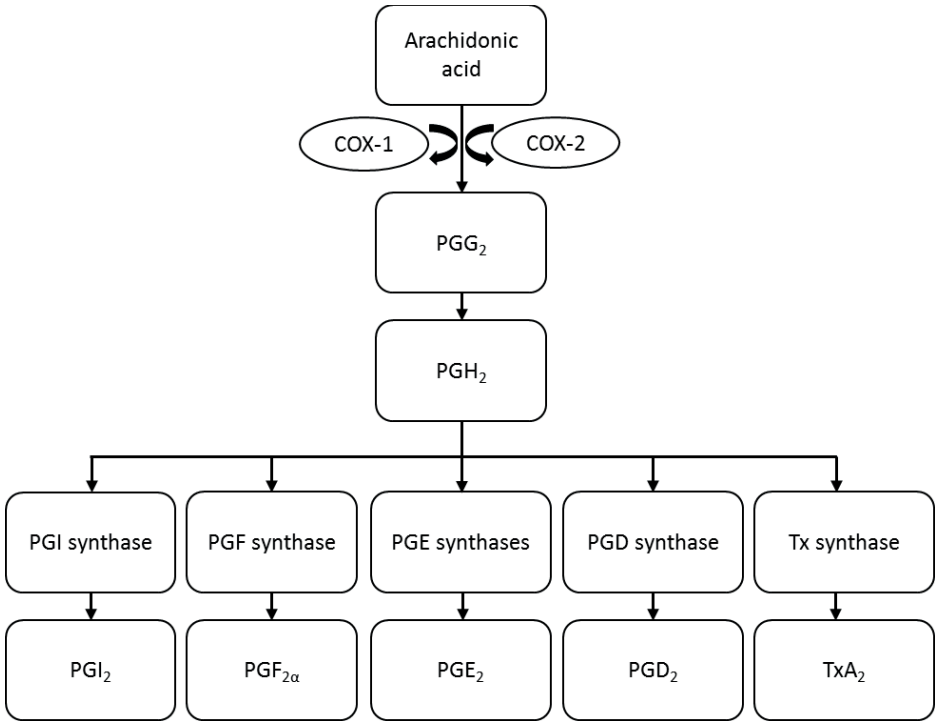


Figure 4. A schematic pathway of prostanoid synthesis. Arachidonic acid is released from cell membrane phospholipids and oxygenated to PG endoperoxide PGG_2 which is converted to PGH_2. Reaction is catalyzed by COX-1 and COX-2. PGH_2 is then metabolized to prostanoids by specific synthases.

Initially arachidonic acid is liberated from membrane-bound phospholipids by phospholipase enzymes, mainly cytosolic phospholipase A_2 ($cPLA_2$). After arachidonic acid has been supplied, both cyclo-oxygenase isoforms, COX-1 and COX-2, catalyze the oxygenation of arachidonic acid to PGG_2 which is further converted to PGH_2 by the same enzyme complex. After these steps, PGH_2 is acted upon by various enzymes to produce different prostanoids. When prostanoids are released, their cellular responses are mediated by specific G-protein coupled prostanoid receptors. (Dennis & Norris 2015, Warner & Mitchell 2004)

Downstream enzymes are PGD synthase, PGF synthase, PGE synthases, PGI synthase and Tx synthase. PGD synthase forms PGD_2 which has two receptors DP1 and DP2. PGD_2 is related to mast cell maturation, vasodilatation, neuroprotection and it increases eosinophil recruitment and allergic response. PGF synthase is found particularly in the uterus and its product $PGF_{2\alpha}$ has a role in uterine, vascular and respiratory smooth muscle contraction and in the reduction of intraocular pressure through its receptor FP. PGI_2 is produced from PGH_2 by PGI synthase. PGI_2 induces vasodilatation and inhibits platelet aggregation, and it is also involved in the mechanisms of hyperalgesia through its IP receptor. Tx synthase forms TXA_2, which increases platelet aggregation and vasoconstriction and inhibits T cell activation. The responses of TXA_2 are mediated by the TP receptor. (Dennis & Norris 2015)

There are three different PGE synthases, namely microsomal PGE synthases 1 and 2 (mPGES-1, mPGES-2) and cytosolic PGES (cPGES) that form PGE_2. mPGES-1 is an inducible enzyme; it was first identified in 1999 by Jakobsson et al. (Jakobsson *et al.* 1999) whereas mPGES-2 and cPGES are constitutively expressed and associated with physiological PGE_2 production. mPGES-1 is induced by many proinflammatory cytokines and it seems to have a role in the pathophysiology of several inflammatory diseases and in cancer. Inhibition of mPGES-1 has been suggested as a potential new target for the drug development to suppress aberrant PGE_2 production. (Korotkova & Jakobsson 2014)

2.4.1 Prostaglandin E_2

PGE_2 was discovered in 1930 by Kurzrok and Leib and it was initially described as a blood pressure–lowering component which could be extracted from the prostate (Kurzrok & Lieb 1930). PGE_2 is a key mediator of inflammation causing swelling, fever and pain. There are four receptors (EP1-4) which all are activated by PGE_2.

Each EP receptor is linked to a different physiological function.(Kawahara *et al.* 2015)

EP1 is related to Th1 differentiation in regulation of contact hypersensitivity (Jia *et al.* 2014). Selective antagonism of the EP1 receptor has been shown to provoke inflammation following intracerebral hemorrhage but EP1 receptor agonism has improved anatomical outcomes and achieved a functional recovery after intracerebral hemorrhage in mice (Leclerc *et al.* 2015). EP2 mediates peripheral hyperalgesia and spinal inflammatory hyperalgesia (Jia *et al.* 2014). Furthermore, the EP2 receptor is expressed in various parts of the body and brain and plays context-dependent, beneficial and harmful roles, which suggests that both agonism and antagonism strategies may be useful routes for therapeutic development. An antagonism strategy could be beneficial in suppressing inflammation and blocking neurodegeneration (Ganesh 2014). In the allergic asthma model, PGE_2 appears to suppress allergic sensitization and lung inflammation through the EP2 receptors in T cells (Kawahara *et al.* 2015). PGE_2 facilitates joint inflammation in arthritis and suppresses dendritic cell differentiation and function via EP2 (Jia *et al.* 2014).

PGE_2 activates mast cell degranulation and cytokine release via the EP3 receptor, resulting in an enhancement of vascular permeability (Kawahara *et al.* 2015). EP3 also mediates fever and suppresses allergic inflammation (Jia *et al.* 2014). EP4 signaling is closely related to carcinogenesis, cardiac hypertrophy, vasodilation, vascular remodeling, bone remodeling, gastrointestinal homeostasis, renal function, and to the classic anti-inflammatory action on mononuclear cells and T cells. Chronic inflammation may also increase EP4 expression. Most of the experiments conducted in macrophages suggest that EP4 is anti-inflammatory because PGE_2 suppresses the production of TNF-α, IL-12, and MCP-1. Nevertheless, there is evidence showing that EP4 receptor signaling also promotes the production of the proinflammatory cytokine, IL-6, in macrophages. Intracellular signaling related to p38 MAPK and NF-κB as well as protein kinase A and protein kinase C are responsible for IL-6 induction by EP4 stimulation. Interestingly EP4-deficient mice, but not EP1-, EP2-, or EP3-deficient mice, have exhibited alleviated inflammation and reduced severity of disease by inhibiting IL-6 and serum amyloid A levels in collagen antibody-induced arthritis. (Yokoyama *et al.* 2013)

3 Osteoarthritis

Osteoarthritis (OA) is the most common form of arthritis. Over 10 % of the adult population has symptomatic OA and it is a leading cause of disability among older adults (>65) (Plotnikoff *et al.* 2015). Because of its high prevalence and the risk of disability, OA is an important disease for both individuals and society. OA inflicts pain, activity limitations and reduced quality of life for individuals and as well direct and indirect costs for the society. Increasing attention should be focused on this disease burden because of societal trends i.e. an ageing population, increased prevalences of obesity and joint injuries indicate that the number of people affected by OA will increase by about 50% over the next 20 years. (Hunter *et al.* 2014, Wang & He 2018)

Knee OA along with hand and hip OA, belongs to the most common forms of OA. The risk of mobility limitations related to knee OA alone is greater than any other medical condition in people aged 65 years and older. The etiology of OA is still unknown but certain risk factors have been identified. Systemic risk factors include genes, gender, age and obesity. In addition, previous joint traumas and overuse of the joint increase the risk of OA. Since there are no effective structure-modifying treatments except surgical interventions, the current treatment options are largely limited to analgesic drugs, which provide a symptomatic relief. (Hunter *et al.* 2014, Wang & He 2018, Wojdasiewicz *et al.* 2014)

3.1 Pathogenesis of osteoarthritis

Joints are complex organs in which different tissues functionally cooperate to allow, while at the same time limiting, the movement between the bones. OA is primarily a disease of articular cartilage but it affects also other joint tissues including subchondral bone, synovium, ligamentous capsular structures, and surrounding muscle and fat. OA is characterized by the progressive loss of articular cartilage with the concomitant joint space narrowing, osteophyte formation, subchondral sclerosis and fluctuating synovial inflammation resulting in pain and loss of joint function and

angular deformity or malalignment. (Liu-Bryan & Terkeltaub 2015, Sofat 2009, Thysen *et al.* 2015)

3.2 Cartilage homeostasis

Cartilage is a key component in synovial joints, and chondrocytes, the only cells present, are surrounded by a dense and highly organized extracellular matrix (ECM). The biochemical properties of cartilage and the physical function of joints are critically dependent on the integrity of this matrix. Under normal conditions, articular chondrocytes maintain a dynamic balance between synthesis and degradation of ECM components. Cartilage ECM is composed of a collagenous network, which mainly contains type II collagen, along with glycosaminoglycans formed of hyaluronan, and a variety of proteoglycans including aggrecan.(Lee *et al.* 2013)

In the chondrocytes of an OA patient, the equilibrium between the synthesis and degradation of cartilage matrix has been disrupted and in advanced OA catabolic processes are accelerated while anabolic processes are suppressed. Upregulation of matrix-degrading enzymes such as matrix metalloproteinases (MMPs), and ADAMTS enzymes (a disintegrin and metalloproteinases with thrombospondin motifs) drive the degradation of cartilage ECM, leading to cartilage destruction. Chondrocyte metabolism is also unbalanced due to the production of inflammatory cytokines like IL-1β, TNF-α and IL-6, chemokines and inflammatory mediators such as COX-2 derived prostanoids and iNOS generated NO in addition to matrix-degrading enzymes. (Kim *et al.* 2015, Liu-Bryan & Terkeltaub 2015)

3.2.1 Cartilage matrix components

In addition to collagens, proteoglycans, hyaluronan and elastic fibers are the major ECM protein components of articular cartilage. Cartilage contains up to 10 % of proteoglycan mostly consisting of aggrecan. Aggrecan contains numerous chondroitin sulfate and keratin sulfate glycosaminoglycan moieties and is sensitive to proteolysis. Aggrecan is important for the proper functioning of articular cartilage because it binds water into the cartilage matrix and forms a hydrated gel structure that provides the cartilage with load-bearing properties.(Kiani *et al.* 2002, Troeberg & Nagase 2012)

The expression of sirtuin 1 (SIRT1) is decreased in human OA cartilage, and inhibition of SIRT1 significantly decreases the expression of aggrecan in both normal and OA human chondrocytes, indicating that histone acetylation may regulate the aggrecan expression (Fujita *et al.* 2011).

Type II collagen is the principal form of collagen present in the articular cartilage; it is a fibrillar collagen that forms a network of fibers where aggrecan molecules are distributed. The tensile strength of cartilage matrix is mostly attributable to this collagen network. Transgenic mice bearing a deletion mutation in the type II collagen gene have been shown to develop OA-like lesions. Collagen degradation is irreversible and cannot be repaired unlike aggrecan loss which can be reversed. Several miRNAs, such as miRNA-146a, -140 and -675 have been found to regulate collagen II expression in articular chondrocytes. There are also other important OA cartilage components e.g. type III, VI, IX, X and XI collagens, biglycan, decorin, fibronectin and tenascin-C. Additionally, SOX-9 is probably the most important transcription factor regulating chondrogenesis. (Poole *et al.* 2002, Sofat 2009, Troeberg & Nagase 2012)

3.2.2 Catabolic factors

The catabolic environment in an OA joint is evident by increases in degradative enzymes and proinflammatory cytokines. Matrix metalloproteinases (MMPs) play a major role in the destruction of the osteoarthritic joint. MMP-1 and MMP-3 are present at high levels in OA synovial fluid. MMP-13 has been associated with high gene expression in OA cartilage. IL-1β stimulates chondrocytes to release several MMPs, including MMP-1, MMP-3, and MMP-13.

In humans, there are known to be 23 members of the MMP family. MMPs are divided into six classes based on the structural and functional characteristics: the collagenases (MMP-1,-8,-13), the gelatinases (MMP-2,-9), the stromelysins (MMP-3,-10,-11), the matrilysins (MMP-7,-26), the membrane type (MMP-14,-15,-16,-17,-24,-25) and others (MMP-12,-19,-20,-22,-23,-27,-28). Although MMP-1, MMP-3 and MMP-13 seem to be the most important MMP enzymes involved in the pathogenesis of OA, other MMPs have been linked to OA e.g. MMP-2,-8 and -9 have also been linked to the disease. (Troeberg & Nagase 2012)

MMPs are regulated by endogenous tissue inhibitors of metalloproteinases (TIMPs). Increased MMP expression or decreased TIMPs could lead to an MMP/TIMP imbalance which results in the development of pathological conditions

like OA. The expressions of MMP-1 and MMP-3 are regulated also via the MAPK signaling pathway. (Cui *et al.* 2017)

MMPs were thought to be the primary aggrecan degrading enzymes until the first aggrecanase ADAMTS-4 (also known as aggrecanase-1) was purified in 1999 by researchers at DuPont Pharmaceuticals (Tortorella *et al.* 1999). The ADAMTS family belongs to the adamalysin group of enzymes and participates in procollagen processing, cleavage of matrix proteoglycans, angiogenesis and the blood coagulation cascade. The importance of ADAMTS in OA was demonstrated in ADAMTS-5$^{-/-}$ mice. These knockout mice developed less severe cartilage damage in surgical and antigen-induced arthritis models. (Tortorella *et al.* 1999, Troeberg & Nagase 2012) An ADAMTS-4 and ADAMTS-5 inhibitor, AGG-523, has been shown to block aggrecan degradation in surgical OA model in the rat (Chockalingam *et al.* 2011). The observations of the destructive effects of MMPs and ADAMTS in OA cartilage have led to the development of inhibitors of these enzymes with the idea being that they will have clinical benefits in OA. ADAMTS-5-selective monoclonal antibody GSK2394002 has been predicted to have potential in clinical studies (Larkin *et al.* 2015) and a clinical study with another ADAMTS-5 inhibitor GLPG1972 in OA has been started based on the clinicaltrials.gov (June 19, 2019), but no results are available.

3.3 Drug treatment of osteoarthritis

Osteoarthritis Research Society International (OARSI) publishes evidence-based, expert consensus recommendations for the treatment of OA. The latest update for the OARSI recommendations was published in 2014. The guideline includes non-pharmacological and pharmacological modalities of treatments. Although it is universally recommended [strength of recommendation (SOR) 96 %, 95 % confidence limits (Cl) 93-99] that the optimal management of OA requires a combination of non-pharmacological and pharmacological treatments, only pharmacological therapies will be examined in this section. (McAlindon *et al.* 2014, Zhang *et al.* 2010)

Paracetamol, at a dose of up to 4 g per day, is the primary recommendation for use as an analgesic mainly because of its safety and efficacy (SOR 92 %). According to recommendations, NSAIDs should be used at the lowest effective dose and long term use should be avoided (SOR 93 %). If the patient carries an increased gastrointestinal risk, either a COX-2 selective NSAID or proton pump inhibitor with

a non-selective NSAID should be used but with caution in patients with cardiovascular risks. Topical NSAIDS and capsaicin can be used together or as an alternative for the oral analgesic in knee OA (SOR 85 %). Topical capsaicin creams first activate and then desensitize peripheral C-nociceptors by activating transient receptor potential vanilloid 1 (TRPV1). Intra-articular injections of corticosteroids can be used in hip or knee OA if the patient has not responded satisfactorily to treatment with an oral analgesic (SOR 78 %). (Mora *et al.* 2018, Zhang *et al.* 2010)

The main differences between the previous guidelines concerning hip and knee OA published in 2010 and in the OARSI 2014 guideline are associated with the use of symptomatic slow-acting drugs like glucosamine and chondroitin and the use of opioids and duloxetine. Based on the recommendation in 2010, glucosamine sulfate, chondroitin sulfate and diacerein (SOR 41 %) and intra-articular injections of hyaluronate (SOR 64 %) were considered useful despite the ongoing controversy of their safety, efficacy and cost-effectiveness. In the 2014 guideline, all these were considered inappropriate based on the benefit and risk score ratio. Furthermore, the use of opioids was considered in 2010 as an option if other pharmacological agents were ineffective or contraindicated (SOR 82 %). In 2014, a recommendation for both transdermal and oral opioids was uncertain because of the risk of serious adverse events. Duloxetine, a serotonin-norepinephrine reuptake inhibitor, was evaluated for the first time in 2014. Based on the data, duloxetine was found to be effective and tolerable for chronic pain related to OA. It was recommended to be appropriate therapy for patients with knee OA only without co-morbidities and patients with multiple-joint OA and relevant co-morbidities. Interestingly, also two natural products rosehip powder and avocado/soybean unsaponifiables were evaluated by the OARSI expert group. Both showed a small benefit for pain but the result was uncertain because of the paucity of large-scale studies. (McAlindon *et al.* 2014, Zhang *et al.* 2010)

Finnish Current Care Guidelines for treatment of knee and hip OA were revised in 2018. In the basic treatment, there are no major differences between the latest OARSI 2014 recommendations and Finnish Current Care Guidelines. The primary treatment in Finnish Current Care Guidelines is paracetamol up to 4 g daily dose and topical NSAIDs with the secondary options being oral NSAIDs. In the Finnish recommendations, opioids are an option if no effect is achieved with paracetamol and NSAIDs or if adverse effects of these first-line drugs preclude their use. Intra-articular injections of corticosteroids and hyaluronate can also be used. (Käypä hoito 2018)

3.4 Drug development for osteoarthritis

As a conclusion from the recommendations of current OA treatments, there are no effective disease-modifying OA drugs (DMOADs) which have received regulatory approval so far. OA is increasingly recognized as a heterogeneous disease with multiple phenotypes involving the whole joint. This means that there are multiple areas in OA for developing potential therapeutic targets including synovium, cartilage, subchondral bone, skeletal muscle and adipose tissue (Figure 5). (Tonge *et al.* 2014)

Despite significant investments in OA research during recent years, there have been difficulties in translating promising effects in animal models into clinical use, with MMP inhibitors being an excellent example of these failures. The development of MMP inhibitors has been limited because of undesirable musculoskeletal adverse effects found in clinical trials of PG-116800 (Laev & Salakhutdinov 2015). Nonetheless, also other candidates ONO-4817, CPA-926 and BMS-561392 have proceeded into clinical trials for the treatment of OA but without published data of the results (Burrage & Brinckerhoff 2007). There still might be hope for MMP inhibitors to be exploited in the treatment of OA but it does seem that more specific inhibitors and additional knowledge about the pathway will be needed. The development of specific MMP inhibitors is challenging because of the extensive structural similarity of the active sites of various MMP enzymes and interconnectivity with other proteases (Vandenbroucke & Libert 2014). Concerns on the safety profiles of MMP inhibitors turned the interest towards inhibitors of aggrecanases. In addition to previously in chapter 3.2.2 mentioned ADAMTS-5 inhibitor GLPG1972, an ADAMTS-4/5 inhibitor, AGG-523, has proceeded to clinical trials but it showed poor pharmacokinetic properties in Phase I studies (Tonge *et al.* 2014).

As an alternative strategy to slow or even prevent cartilage destruction, fibroblast growth factors FGF-2 and FGF-18 have been recognized as targets as they activate anabolic pathways. Several candidates are currently undergoing Phase I/II trials (Tonge *et al.* 2014, Watt & Gulati 2017). An early placebo-controlled trial of intra-articular FGF-18 in patients with knee OA showed disappointing results (Chevalier *et al.* 2013). In contrast, promising results of a clinical trial investigating intra-articular sprifermin, a recombinant human FGF-18, have been published (Lohmander *et al.* 2014). The β-nerve growth factor (β-NGF) antibody, tanezumab, administered by intravenous injections has been able to reduce joint pain and improve physical function in patients with knee OA, but at the same time, it caused neurological adverse effects and when administered together with NSAIDS, it was associated with

cases of rapid progressive OA (Chevalier *et al.* 2013, Watt & Gulati 2017, Yuan *et al.* 2014).

There have been expectations that biological agents used in the treatment of rheumatoid diseases would be beneficial in the treatment of osteoarthritis but these have not so far been realized. The TNF antagonist, infliximab, has been studied in the treatment of patients with hand OA and knee OA without significant results. However, the results of a small study with the TNF antagonist, adalimumab, in patients with knee OA with synovial effusion showed that the numbers of new erosions were significantly decreased, suggesting that patients with intense inflammation might benefit from this treatment. Results from clinical trials examining IL-1β inhibition have been negative in the treatment of OA but IL-1β blockade might be beneficial for controlling the inflammatory process after traumatic knee injuries that precede the development of OA. IL-6 is a pro-inflammatory and catabolic cytokine detected in synovial fluid and expressed in OA cartilage; for this reason, IL-6 inhibition is an appealing potential target not least because of its ability to regulate the activities of ADAMTS-4 and ADAMTS-5. At the moment, no studies with IL-6 inhibitors and OA have been conducted but based on a search in clinicaltrials.gov (June 19, 2019) one trial has been planned to examine the effect of tocilizumab in patients with hand OA. (Chevalier *et al.* 2013, Dancevic & McCulloch 2014, Glyn-Jones *et al.* 2015)

Bisphosphonates have been candidates to treat the subchondral bone changes seen in OA because of their ability to inhibit osteoclasts. Unfortunately, no disease-modifying effects have been seen in humans with bisphosphonate treatment. Strontium ranelate can decrease bone resorption and increase bone formation and it has shown some positive effects in clinical trials. Statins are of interest because of their anti-inflammatory actions but further studies are needed to evaluate their effects. (Dancevic & McCulloch 2014, Yuan *et al.* 2014)

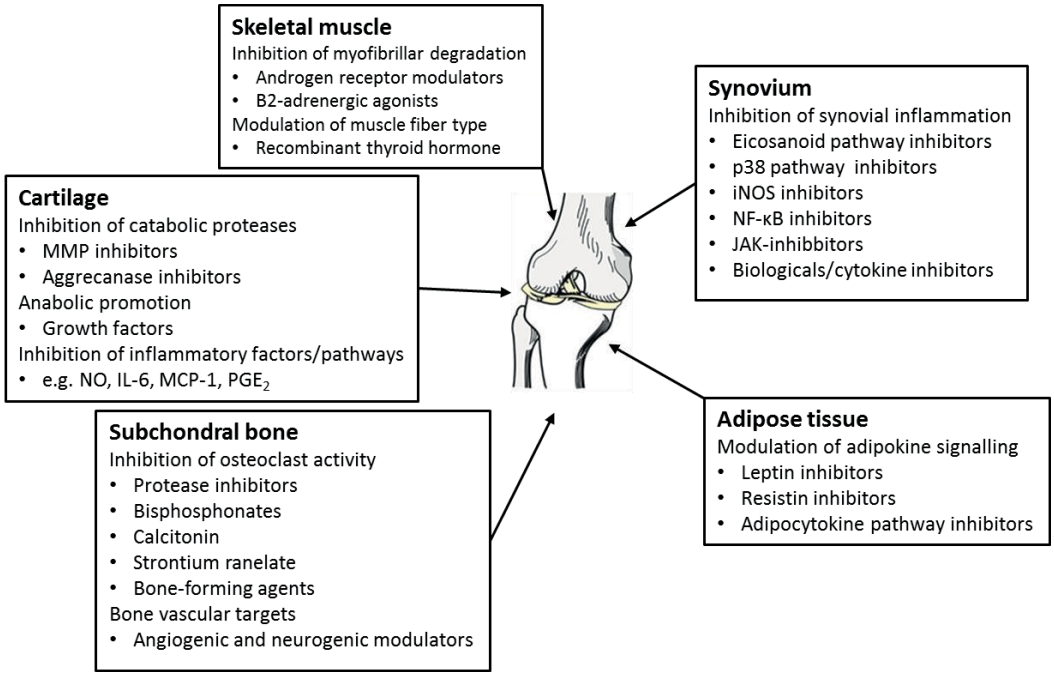


Figure 5. Possible drug targets for the development of DMOADs. Modified from (Tonge *et al.* 2014)

There is also growing interest in herbal medicines in the treatment of OA. This might be due to increased patient-driven search for alternative treatments or because of frustration with the fact that modern scientific medicine has not been able to treat the disease. In addition, that is an increasing tendency also in pharmaceutical industry as will be discussed in chapter 4. Compounds derived from plants that can modulate the expression of pro-inflammatory cytokines, catabolic and anabolic factors or cartilage matric components, have shown some potential against OA. Although there are no large-scale clinical trials which would have confirmed the potential for using natural products (Laev & Salakhutdinov 2015, Mobasheri 2012)

In the future, advanced personalized approaches are needed for the drug development for OA. OA has several disease phenotypes – for this reason, the identification and specific targeting of the phenotypes should be taken into account. Clinical trials investigating the efficacy of an intervention that intended to target a particular feature of the disease pathogenesis, might be more likely to achieve positive results in a targeted patient group than in large unselected populations since only a small subset of these patients might have a disease that is driven by this particular feature. (Glyn-Jones *et al.* 2015)

4 Natural products as part of the drug discovery

Throughout the ages, people have sought cures from nature to treat their symptoms and diseases. In particular, plants have been the foundation stones of classic traditional medicine. The earliest records of sophisticated medicine systems are documented in Mesopotamia around 2600 BCE and the most well-known record "Ebers Papyrus" which dates from 1500 BCE describes over 700 drugs, mostly of plant origin. Similarly, traditional Chinese medicine has been widely documented over the years with the first record dating to about 1100 BCE. (Cragg & Newman 2001)

Traditional medicine has been a fertile source for drug discovery throughout the centuries and plant-based systems still play an important role in healthcare (Cragg & Newman 2013). Natural products, chemical compounds produced by a living organism, were first utilized as plant extracts but with the advancement of chemistry and drug formulations, it has become more common that a purified compound is used (Mishra & Tiwari 2011). Good examples of traditional medicine guiding drug discovery and development are antimalarial drugs the quinine and artemisinin. Quinine was isolated from the bark of *Cinchona* species which had long been used in the Amazon region to treat fevers. Quinine was the basis for the synthesis of cloroquine and mefloquine which subsequently replaced quinine. When resistance for these drugs increased in many areas, another plant *Artemisia annua* used in traditional Chinese medicine provided a new lead compound - artemisinin. (Cragg & Newman 2013)

The introduction of high-throughput synthesis and combinatorial chemistry in 1980s reduced the screening of natural products by the pharmaceutical industry. Combinatorial chemistry´s potential to deliver a large number of novel compounds in a very short time led some companies to terminate their natural products screening programs. Natural products discovery was associated with several problems: expense, time, novelty, tractability, scale-up and intellectual property. Building up collections of natural product extracts is expensive and once a hit is identified, it is possible that the lead may turn out to be an already known natural product which cannot be patented. Meaningful structure-activity relationships can be difficult to identify, even obtaining further quantities of compounds can be limited. Increasing

protection related to biodiversity may also lead to complex negotiations. (Ortholand & Ganesan 2004, Stratton *et al.* 2015)

The declining numbers of new drug approvals have led to a reassessment of the policy to exclude natural products in drug discovery (Newman & Cragg 2016, Ortholand & Ganesan 2004). There has been a trend over the last 30 years that the numbers of small-molecule drugs have decreased whereas there have been increases in biologic therapeutics like antibodies and vaccines (Stratton *et al.* 2015). Nonetheless, small-molecule drugs remain an important component of the drug development pipeline. In fact, the year 2004 was the worst in the period 1981-2014, only 24 new drug entities were registered but 7 of them (29 %) were naturally derived (Newman & Cragg 2016). Between January 1st 1981 and December 31st 2014, a total of 1562 new drugs were approved worldwide (Newman & Cragg 2016). The sources of these new drugs can be classified into eight categories, see Table 6.

Table 6. Categories of the new drugs according to their sources (modified from Newman & Cragg 2016)

Category	Source of the drug
B	Biological; usually a large (>45 residues) peptide or protein isolated from an organism/cell line or produced by biotechnological means in a surrogate host.
N	Unaltered natural product
NB	Natural product "Botanical mixture"
ND	Derived from a natural product and is usually a semi-synthetic modification
S	Totally synthetic drug, often found by random screening/modification of an existing agent
S*	Made by total synthesis, but the pharmacophore is/was from a natural product
V	Vaccine
/NM	Mimic of natural product (direct competitive inhibitors of the natural substrate)

From Figure 6, it can be seen that 30 % of all entities during this 33 year period have originated from natural sources (N, NB, ND, S*) with this value rising up to 51 % if natural mimics are also included (N, NB, ND, S*,S/NM, S*/NM).

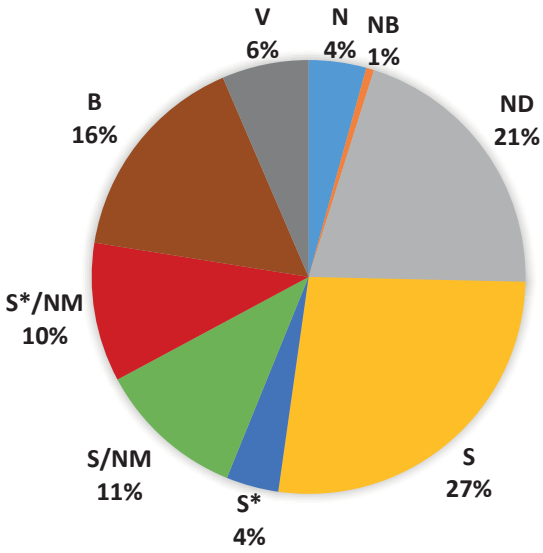


Figure 6. New drugs during 1981-2014 according to their sources (modified from Newman & Cragg, 2016) – see Table 6 for explanation of the letters above the percentages

If only small-molecule drugs are considered, 65 % of them have their inspiration in nature (Figure 7). If it is desired to reverse the decreasing trend of new drug entities, a multidisciplinary approach is needed in drug development. Combinatorial chemistry is an extremely powerful tool for the optimization of an active natural product's structure; it needs to be combined with the expertise of synthetic chemists to improve the adsorption, distribution, metabolism and excretion properties of natural products.

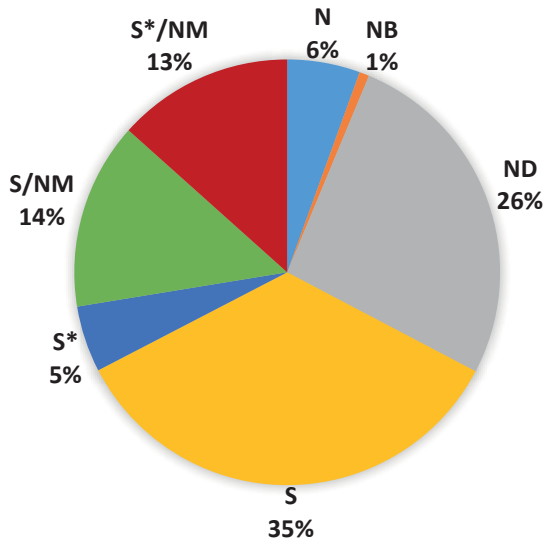


Figure 7. New small-molecule drugs during 1981-2014 according to their sources (modified from Newman & Cragg 2016) – see Table 6 for explanation of the letters above the percentages

Traditionally, especially anti-cancer and anti-microbial drugs have been derived from nature but there are also good examples of immunological and anti-inflammatory products. For example, the immunosuppressive drug, cyclosporine, which originally was derived from the filamentous fungus *Tolypocladium inflatum,* revolutionized organ transplantation in 1983, followed ten years later by tacrolimus extracted from a soil bacterium named *Streptomyces tsukubaensis* (Azzi *et al.* 2013, Mishra & Tiwari 2011).

Among the other natural sources, trees have played an important role in drug discovery based on traditional medicine. The most famous story is the development of the NSAID, acetylsalicylic acid, marketed as Aspirin® from salicin in the willow bark. Chinese healers were using willow bark at least in 500 BCE to treat several illnesses and in Greece, Hippocrates (430-377 BCE) used it for his patients, especially to treat pain and reduce fever. In 1899, acetylsalicylic acid was patented by Bayer and it became the most popular painkiller around the world. In 1980s, Aspirin® became also a standard treatment for cardiovascular patients to prevent ischemic stroke and unstable angina pectoris. (Ugurlucan *et al.* 2012)

The taxanes represent another success story; these agents are used in the treatment of breast cancer. Paclitaxel, isolated from extracts from Pacific yew trees

(*Taxus brevifolia*), was noted to possess antitumor activity in the 1960s and subsequently in animal models for melanoma, breast, lung, and colon cancers. A more potent semisynthetic derivative, docetaxel, synthetically modified from the needles of the European yew tree (*Taxus baccata*), was subsequently discovered in the 1980s. (Gradishar 2012)

Trees are rich in polyphenolic compounds and could still be potential sources for novel drugs. Knots, i.e. the part of the branches embedded in the stem, also called branch roots, are known to be a rich source of several polyphenols, such as lignans, flavonoids and stilbenes (Holmbom *et al.* 2007). Pine wood resin also contains terpenes (Nuopponen *et al.* 2004). In traditional medicine, pine oil and extract have been used to repel parasites, insects and bacteria, and to treat respiratory diseases, urinary tract infections, arthritis, rheumatism, gout and wounds. Furthermore, pine needles were used by ancient Romans and Greeks to treat respiratory problems and muscular pain (Darshan & Doreswamy 2004, Suntar *et al.* 2012).

It has been shown that *Pinus sylvestris* leaf buds had promising anti-inflammatory effects by reducing NO production and iNOS mRNA expression in LPS and IFN-γ stimulated murine macrophages even though the extracts exerted no effects on COX-2 expression or PGE_2 production (Karonen *et al.* 2004). Nonetheless, the essential oils obtained from the needles of *Pinus sylvestris* did not have any significant effect on the wound healing process (Suntar *et al.* 2012).

4.1 Stilbenoids

Stilbenoids are hydroxylated derivatives of *trans*-stilbene (Figure 8) which have a C6-C2-C6 structure. *Trans*-isomers are usually more dominant in plants. Stilbenoids are part of our normal diet. These compounds can be found in grapes, almonds, rhubarb and berries. Stilbenoids are also secondary products of heartwood formation in trees where they act as phytoalexins. Two stilbenoids, pinosylvin and monomethylpinosylvin, have been isolated from the knots of Scots pine (*Pinus Sylvestris*) (Willför *et al.* 2003). The most widely investigated bioactive stilbenoid is resveratrol. In addition, some other natural substances from the stilbenoid group i.e. cis-mulberroside A, rhapontigenin, pterostilbene and piceatannol have been demonstrated to have biological activities (Roupe *et al.* 2006, Song *et al.* 2015, Zhang & Shi 2010, Zhou *et al.* 2015). The structures of the stilbenoids examined in this study are presented in Figure 9.

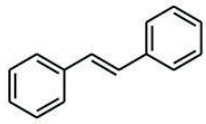


Figure 8. Basic C6-C2-C6 structure of stilbene

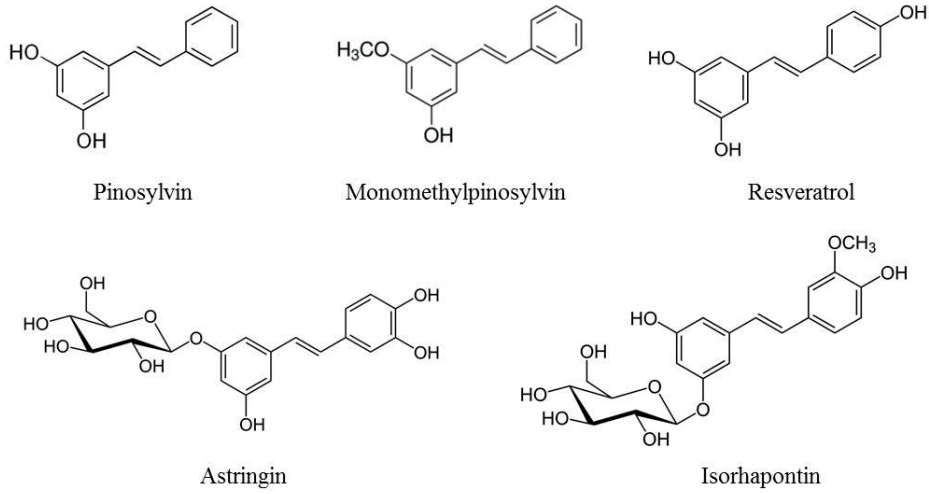


Figure 9. Structures of the stilbenoids used in this study

4.1.1 Resveratrol

Resveratrol was first isolated in 1939 from *Veratrum grandiflorum,* the root of the white hellebore by Takaoka (Takaoka 1939). Interest in resveratrol expanded after the article "the French paradox" was published in 1992 (Renaud & de Lorgeril 1992). The article suggested that it could be wine consumption that was protecting people in France from coronary heart disease despite the relatively high consumption of saturated fat (Renaud & de Lorgeril 1992). Later, resveratrol was identified as an ingredient explaining the possible health protective properties in red wine. Actually, the concentration of resveratrol is relatively low in wine (average 1.9±1.7 mg/l) and in addition to resveratrol, these grapes contain over 1600 other phytochemicals (Park & Pezzuto 2015, Stervbo *et al.* 2007).

The interest in resveratrol became intense and over 12 000 publications can now be found in PubMed. Resveratrol has protective effects related to the cardiovascular

system, inflammation, energy metabolism, obesity and cancer (Poulsen *et al.* 2015). Resveratrol has also attracted significant attention because of its possible ability to extend lifespan or provide protection against age-related diseases (Bhullar & Hubbard 2015). This line of research started after resveratrol had been found to activate SIRT1 and to extend the lifespan of yeasts, worms and flies (Bhullar & Hubbard 2015, Howitz *et al.* 2003). SIRT1 regulates many cellular functions including DNA repair, fat cell differentiation, glucose output, insulin sensitivity, fatty acid oxidation and neurogenesis. However, it is not clear if SIRT1 is the direct target of resveratrol, in fact resveratrol targets a number of kinases and other enzymes, and receptors related to the conditions listed above (Borra *et al.* 2005, Pacholec *et al.* 2010).

There are already commercial products e.g. nutraceuticals and cosmetics in the market containing resveratrol. A variety of dietary supplements containing resveratrol from 0.2 to 1000 mg per dose are available as a single component or in combination with other ingredients. (Park & Pezzuto 2015)

4.1.1.1 Anti-inflammatory effects in animal models

The effects of resveratrol have been studied in several animal models of inflammatory diseases and resveratrol has been shown to downregulate many inflammation induced biomarkers. This section concentrates on the studies published in the years 2010-2015, which are shown in Table 7. Interestingly, resveratrol has been tested in three different OA models. Two of them were surgical models, one in mouse and one in rabbit and the third was conducted in mice where OA was induced by collagen. Based on these studies, resveratrol seems to prevent cartilage destruction, bone erosion and synovial hyperplasia. It has been reported to suppress the production of the catabolic factor MMP-13 and the expression of the inflammatory enzyme iNOS. It also increased the production of the cartilage specific type II collagen and decreased proteoglycan loss, indicative of beneficial properties in the treatment of OA. (Li *et al.* 2015, Wang *et al.* 2012, Zou *et al.* 2013)

In addition, there is one study concerning gouty arthritis induced by monosodium urate crystals, where joint swelling was decreased as well as the expressions of the proinflammatory cytokine IL-1β, the chemokine CXCL10, the chemokine receptor CCR5 and the inflammasome component NLRP3 (Wang *et al.* 2015). Resveratrol reduced the skinfold thickening and erythema scaling scores in psoriasis-like skin inflammation, possibly by decreasing IL-17A, IL-17F and IL-19 (Kjaer *et al.* 2015). In two studies, beneficial effects were achieved in models of acute pancreatitis (Jha

et al. 2012, Sha *et al.* 2013). Resveratrol administration in different intestinal inflammation models has resulted in extended survival, positive effects on weight loss and downregulation of proinflammatory IL-1β, IL-6, TNF-α, MCP-1 and upregulation of the anti-inflammatory, IL-10 (Bereswill *et al.* 2010, Rahal *et al.* 2012, Sanchez-Fidalgo *et al.* 2010).

Table 7. Summary of anti-inflammatory effects of resveratrol in animal models

Species	Dose	Route	Duration	Model	Outcome	Reference
Male C57BL/6 mice	15 mg/kg, once	i.p.	6 hours (treatment 1 h after MSU)	Monosodium urate (MSU) crystal induced gouty arthritis	Joint swelling ↓ Inflammatory cell infiltration ↓ IL-1β ↓ TNF-α ↓	(Li *et al.* 2019)
Male Sprague-Dawley rats	30 mg/kg, twice	i.p.	18 hours (treatment 6 h and 12 h after operation)	Cecal ligation and puncture (CLP) model of peritonitis	Blood urea nitrogen ↓ CRP ↓ IL-1β ↓ TNF-α ↓ Nrf2 ↓ HO-1 ↑ Kidney injury molecule-1 ↓	(Wang *et al.* 2018)
Female BALBc mice	3 mg/kg, twice	i.p.	19 days (treatment on day 15 of pregnancy)	LPS-induced preterm labor	Preterm labor prevention ↑ iNOS ↓ COX-2 ↓	(Bariani *et al.* 2017)
Mice	30, 10, and 3 mg/kg daily	oral	7 days	Xylene-induced mouse ear oedema	Ear oedema ↓	(Wang *et al.* 2017)
Sprague-Dawley rats	30, 10, and 3 mg/kg daily	oral	7 days	Carrageenan-induced synovitis	White blood cell count ↓ Thromboxane receptor ↓ PGE_2 ↓ NO ↓ Malondialdehyde ↓	(Wang *et al.* 2017)
Male C57BL/6 mice	10 μg or 100 μg once a week	Intra articular	8 weeks (treatment 4 weeks)	Surgically induced osteoarthritis	Articular cartilage destruction ↓ Thickness of calcified cartilage ↑ Mankin score improvements MMP-13↓ iNOS ↓ Collagen II ↑	(Li *et al.* 2015)

Species	Dose	Route	Duration	Model	Outcome	Reference
Male BALBc/AnNTac mice	400 mg/kg/day	In diet	7 days	Imiquimod induced psoriasis-like skin inflammation	Psoriasis Area Severity Index ↓ Erythema score ↓ Skin thickness ↓ Epidermal thickness ↓ IL-17A ↓ IL-19 ↓ Protein kinase C1 ↑ TRIM63 ↑ Protein phosphatase 1 regulatory subunit 3C (PPP1R3C) ↓	(Kjaer *et al.* 2015)
Male SPF mice	500 mg/kg/day	Gavage	72 hours (treatment during 4 days before MSU)	Monosodium urate (MSU) induced acute gouty arthritis	Joint swelling ↓ IL-1β ↓ CCR5 ↓ CXCL10 ↓ NLRP3 ↓	(Wang *et al.* 2015)
Male C57BL/6 mice	10, 20 and 40 mg/kg/day	i.p.	5 hours (treatment 1 hour before mechanical ventilation)	Mechanical ventilation-induced inflammation	Pulmonary NF-κB activity ↓ Lung: IL-1β, IL-6, KC ↔ Plasma: TNF-α, KC, IL-6 ↔	(Van der Wal *et al.* 2014)
Male DBA1 mice	20 mg/kg/day	Gavage	8 weeks, daily treatment	Collagen-induced arthritis	Incidence and severity of arthritis ↓ Infiltrated cells in the joint ↓ Synovial hyperplasia ↓ Bone erosion ↓	(Zou *et al.* 2013)
Sprague-Dawley rats	20 mg/kg, once	i.v.	3, 6, 12 hours	Taurocholate-induced severe acute pancreatitis	Histopathologic changes ↓ Renin activity ↓ Angiotensin II ↓ Endothelin ↓ NO ↓	(Sha *et al.* 2013)
Male and female New Zealand white rabbit	10, 20 and 50 μmol/kg, daily	Intra articular	6 weeks and 4 days (treatment 2 weeks)	Surgically induced osteoarthritis	Cartilage destruction ↓ Mankin score improvements NO ↓ Proteoglycan loss ↓ Apoptosis rate ↓	(Wang *et al.* 2012)

Species	Dose	Route	Duration	Model	Outcome	Reference
Female Lewis rats	100 mg/kg/day	Gavage	28 days, daily	Peptidoglycan-polysaccharide model of Crohn's disease	Histologic fibrosis score in cecal tissue ↓ IGF-1, procollagen type III ↔ IL-1β ↓ TNF-α ↓ IL-6 ↓ TGF-β1 ↓	(Rahal *et al.* 2012)
Male Sprague-Dawley rats	20 mg/kg, once	i.v.	3, 6, 12 hours	Taurocholate-induced severe acute pancreatitis	Superoxide dismutase ↑ Malondialdehyde ↓ TNF-α ↓ ICAM-1 ↓ VCAM-1 ↓	(Jha *et al.* 2012)
Female C57BL/6 mice	3 mg/kg/day	In diet	3 weeks	Induction of chronic colitis by dextran sulfate sodium	Extended survival Loss of body weight, diarrhea and rectal bleeding ↓ IL-1β ↓ TNF-α ↓ PGES-1 ↓ COX-2 ↓ iNOS ↓ IL-10 ↑	(Sanchez-Fidalgo *et al.* 2010)
C57BL/10ScSn wild type mice	20, 100, 200mg/kg/day, daily	Gavage	10 days (treatment 2 days before *T.gondii*)	*Toxoplasma gondii* induced acute small intestinal inflammation	Extended survival Loss of body weight, small intestinal shortening ↓ Regeneration in the ileum mucosa ↑ Number of regulatory T cells ↑ Number of mucosal T lymphocyte and neutrophilic granulocytes ↓ IL-10 ↑ IL-23p19 ↓ IFN-γ ↓ TNF-α ↓ IL-6 ↓ MCP-1 ↓	(Bereswill et al. 2010)

4.1.1.2 Anti-inflammatory potential in clinical studies

Although the findings in pre-clinical studies have been promising, there have been some difficulties in translating these results into patients (Poulsen *et al.* 2015). Based on a search (conducted on January 27, 2019) in clinicaltrials.gov, 152 clinical studies are listed related to resveratrol. Several of them are related to inflammation but no studies were found evaluating the effects of resveratrol in patients with osteoarthritis or rheumatoid arthritis. The clinical trials investigating the anti-inflammatory potential of resveratrol in which there has been a positive outcome are listed in Table 8. However, only one of these studies (Samsami-kor et al. 2015) was investigating the anti-inflammatory potential as the main target. In addition to the trials listed in Table 8, a couple of other studies have been published where no anti-inflammatory effects were found (Poulsen *et al.* 2013, Semba *et al.* 2014, Yoshino *et al.* 2012).

Table 8. Human clinical trials indicating positive outcome in investigating anti-inflammatory potential of resveratrol

Study	Design	Outcome
(Khojah *et al.* 2018)	Randomized, placebo controlled study 1 g resveratrol co-administered with DMARDs vs. placebo daily for 3 months N=100 RA patients	Serum C-reactive protein (CRP) ↓ Serum TNF-α ↓ Serum IL-6 ↓ Serum MMP-3 ↓
(Lv *et al.* 2018)	Double-blind, randomized, placebo controlled study 2 sprays (100 μL/spray) in each nostril 3 times/day for 1 month N=100 allergic rhinitis patients	Eosinophils ↓ IL-4 ↓ TNF-α ↓ IgE ↓
(Moussa *et al.* 2017)	Double-blind, randomized, placebo controlled study 500 mg resveratrol vs. placebo daily for 13 weeks and then 1 g twice daily totally 52 weeks N=119 Alzheimer´s disease patients	Plasma IL-1R4 ↓ Plasma IL-12P40 ↓ Plasma IL-12P70 ↓ Plasma TNF-α ↓ Plasma RANTES↓ Plasma MMP-10 ↑

Study	Design	Outcome
(Samsami-Kor *et al.* 2015)	Double-blind, randomized, placebo controlled study 500 mg resveratrol vs. placebo daily for 6 weeks N=50 ulcerative colitis patients	Inflammatory bowel disease questionnaire-9 ↑ Simple colitis clinical activity index ↓ CRP ↓ Serum TNF-α ↓ PBMC NF-κB p65 ↓
(Faghihzadeh *et al.* 2014)	Double-blind, randomized, placebo controlled study 500 mg resveratrol vs. placebo daily for 12 weeks N=50 patients with nonalcoholic fatty liver disease	Serum alanine aminotransferase ↓ Serum cytokeratin-18 ↓ Serum CRP ↓ Serum IL-6 ↓ PBMC NF-κB p65 ↓ Serum TNF-α ↔
(Witte *et al.* 2014)	Double-blind, randomized, placebo controlled study 200 mg resveratrol+320 mg quercetin vs. placebo for 26 weeks N=46 healthy obese older people	Functional connectivity of hippocampus ↑ Memory retention (auditory verbal learning test) ↑ Serum CRP ↔ Serum leptin ↑ Serum IL-6 ↓ Serum TNF-α ↓
(Militaru *et al.* 2013)	Double-blind, randomized, active-controlled, parallel study 20 mg resveratrol, 20 mg resveratrol+112 mg calcium fructoborate, 112 mg calcium fructoborate, control for 60 days N=116, patients with stable angina pectoris	Serum CRP ↓ Positive effects on lipid profile and left ventricular function markers
(Agarwal *et al.* 2013)	Double-blind, randomized, parallel, placebo controlled study 400 mg resveratrol, 400 mg grape skin extract, 100 mg quercetin, placebo for 30 days N=44 healthy people	Plasma IFN-γ ↓ Plasma IL-8 ↓ Plasma ICAM ↓ Plasma VCAM ↓ Plasma IL-1β ↔ Plasma IL-6 ↔ Plasma TNF-α ↔ Plasma leptin ↔

Study	Design	Outcome
(Bo *et al.* 2013)	Double-blind, randomized, placebo controlled, cross-over study 500 mg resveratrol for 30 days, 30 days wash-out, 30 days placebo and vice versa N=50 healthy adult smokers	Serum CRP ↓ Triglycerides ↓ Total antioxidant status ↑
(Zahedi *et al.* 2013)	Double-blind, randomized, placebo controlled study 40 mg resveratrol in *Polygonum cuspidatum* extract vs. placebo daily for 6 weeks N=20 healthy male professional basketball players	Serum IL-6 ↓ Serum TNF-α ↓
(Tome-Carneiro *et al.* 2013)	Triple-blind, randomized, parallel, dose-response, placebo controlled study 8 mg resveratrol in grape supplement, grape supplement, placebo daily for 6 months and double doses for another 6 months N=75 males with cardiovascular risk factors	Serum CRP ↓ Serum IL-6 ↔ Serum IL-6/IL-10 ratio ↓ Serum IL-10 ↑ Serum TNF-α ↓ Plasminogen activator inhibitor 1 ↓ Decreasing trend in ICAM-1 and IL-18
(Brasnyo *et al.* 2011)	Double-blind, randomized, placebo controlled study 5 mg x 2 resveratrol vs. placebo daily for 30 days N=19 type 2 diabetic patients	Oxidative stress ↓

Study	Design	Outcome
(Timmers *et al.* 2011)	Double-blind, randomized, placebo controlled, cross-over study 150 mg resveratrol for 30 days, 4 weeks wash-out, placebo for 30 days N=11 obese men	Plasma TNF-α ↓ Number of leucocytes ↓ Leptin ↓ Glucose, insulin and insulin resistance index ↓ Decreasing trend in IL-6 and CRP
(Bakker *et al.* 2010)	Double-blind, randomized, placebo controlled, cross-over study Dietary mix containing 6.3 mg resveratrol, placebo N=36 healthy obese men	Serum CRP ↔ Plasma IL-18 ↓ Plasma ICAM-1 ↓ Plasma VCAM-1 ↓ Plasma CC-chemokine ligand CCL22 ↓ Adiponectin ↑
(Ghanim *et al.* 2010)	Double-blind, randomized, placebo controlled study 40 mg resveratrol in *Polygonum cuspidatum* extract vs. placebo daily for 6 weeks N=20 healthy people	Oxidative stress ↓ Serum CRP ↓ Plasma IL-6 ↓ Plasma TNF-α ↓ PBMC NF-κB ↓ PBMC β-catenin kinase (IKKβ) ↓ PBMC suppressor of cytokine signaling 3 (SOCS-3) ↓ PBMC c-Jun N-terminal kinase-1 (JNK-1) ↓

In 2010, resveratrol was a component in a dietary mix or a plant extract in two clinical studies investigating inflammatory factors (Bakker *et al.* 2010, Ghanim *et al.* 2010). The first double-blind randomized placebo controlled clinical study examining in a systematic manner the metabolic and anti-inflammatory effects of resveratrol in humans was published by Timmers et al. in 2011. A small decrease in the concentrations of the proinflammatory cytokine TNF-α and a trend towards lower levels IL-6 and CRP were found (Timmers *et al.* 2011). The reduced IL-6 serum or plasma levels are supported by four other studies investigating resveratrol (Faghihzadeh *et al.* 2014, Ghanim *et al.* 2010, Witte *et al.* 2014, Zahedi *et al.* 2013). The reduction in TNF-α levels are also supported by five studies in addition to Timmers et al. but there are also trials where no changes in TNF-α were seen (Ghanim *et al.* 2010, Samsami-Kor *et al.* 2015, Tome-Carneiro *et al.* 2013, Witte *et al.* 2014, Zahedi *et al.* 2013). Interestingly, in 2013 Tome-Carneiro et al. investigated peripheral blood mononuclear cells (PBMCs) from patients taking the grape-

resveratrol extract, and found that the levels of a group of miRNAs (miR-21, miR-181b, miR-663, miR-30c2, miR-155 and miR-34a) involved in the regulation of the inflammatory response were altered. Changes in serum CRP have been contradictory in several studies. Possible explanations of the conflicting results can be found from differences in study populations, doses, length of treatments and the health status of participants. Future large-scale studies focusing on the anti-inflammatory effects as the primary outcome and administering pure resveratrol are needed to find a consensus about the correct dosing and to deliver convincing evidence of its putative anti-inflammatory properties. (Poulsen *et al.* 2015)

4.1.1.3 Pharmacological mechanisms

Adenosine monophosphate-activated kinase (AMPK) and SIRT1 have been claimed to be the key metabolic effectors of the health benefits of resveratrol (Baur *et al.* 2006, Howitz *et al.* 2003). It has been suggested that AMPK and SIRT1 might share a common signaling pathway. There is evidence that resveratrol could activate AMPK which could lead to a downstream activation of SIRT1. AMPK activation increases nicotinamide adenine dinucleotide (NAD^+) and this would favor SIRT1 activity (Kulkarni & Canto 2014). For example, mitochondrial biogenesis induced by resveratrol can be prevented by blocking either AMPK or SIRT1 (Price *et al.* 2012, Um *et al.* 2010).

From anti-inflammatory perspective, other possible effectors of resveratrol could be cyclo-oxygenases, NF-κB, PI3K/Akt or MAPK signaling. Resveratrol can directly inhibit COX-1 and COX-2 activity but COX activity can also be reduced by transcriptional mechanism by downregulating Akt, MAPK or NF-κB (Kulkarni & Canto 2014).

Resveratrol interferes with transcription factors belonging to the NF-κB family. Resveratrol has been repeatedly reported to reduce NF-κB activity but the mechanisms lying behind these properties are not clear. It has been postulated that resveratrol suppresses IκB kinase activity and would therefore prevent IκB degradation and NF-κB translocation. (Ren *et al.* 2013)

PI3Ks are a family of enzymes involved in many cellular functions but also in inflammation. Resveratrol inhibits the phosphorylation of Akt, which is a widely used marker to reflect PI3K activity (Eräsalo *et al.* 2018, Fröjdö *et al.* 2007, Kimbrough *et al.* 2015). Resveratrol is known to decrease at least class IA PI3K catalytic subunits p110α and p110β, which are suggested to regulate more generally growth and survival than inflammation (Fröjdö *et al.* 2007).

Some biological activities of resveratrol have been related to the MAPK subfamilies ERK1/2, p38 and c-Jun N-terminal kinase (JNK). The effects are suppressing or activating depending on the dosage (Pervaiz & Holme 2009). There is also one study suggesting that inhibition of phosphodiesterases could be involved in the metabolic benefits of resveratrol. Resveratrol inhibited phosphodiesterases (PDE) 1, 3 and 4 with IC_{50} values of 6-14 μM. Interestingly, in the same study, the PDE4 inhibitor rolipram had similar metabolic effects as resveratrol in mice (Park *et al.* 2012). The anti-inflammatory effects of PDE4 inhibitors have been shown to be at least partly mediated by MKP-1, a subgroup of the MAPK phosphatases (Korhonen *et al.* 2013).

4.1.2 Pinosylvin and Monomethylpinosylvin

Some biological activities of pinosylvin have been investigated in previous years. Less is known about monomethylpinosylvin, also called methylpinosylvin and pinosylvin monomethyl ether. In trees, pinosylvin exerts antibacterial and antifungal properties (Välimaa *et al.* 2007). There are few studies on the antioxidant, chemopreventive, immunomodulatory, and nociceptive effects of pinosylvin.

4.1.2.1 Immunomodulatory, antioxidant and antinociceptive properties

Pinosylvin was initially reported to have anti-inflammatory activities in 2004 when it was shown to inhibit LPS induced PGE_2 production in mouse macrophages (RAW.263) (Park *et al.* 2004). Pinosylvin also suppressed LPS induced expression of IL-8 and TNF-α in THP-1 cells via the NF-κB signaling pathway (Lee *et al.* 2006). In 2011, Park et al. reported that pinosylvin inhibited iNOS protein and mRNA expression and NO production in RAW.263 macrophages (Park *et al.* 2011).

The adjuvant arthritis model has been so far the only inflammatory model used in *in vivo* studies with pinosylvin. Pinosylvin treatment was found to inhibit hind paw volume and to decrease the activity of cellular γ-glutamyltransferase in the joint and to reduce the number of neutrophils in blood (Drafi *et al.* 2012, Jancinova *et al.* 2012, Macickova *et al.* 2010). Quite recently, a report was published where pinosylvin was tested alone and in combination with methotrexate in the same model. Pinosylvin significantly suppressed the activation of NF-κB in liver and lungs. It also improved HO-1 expression and the activity of lipoxygenase in the lung and decreased MCP-1 and F2-isoprostane levels in plasma (Bauerova *et al.* 2015).

An interesting new finding related to the anti-oxidant properties of pinosylvin showed that pinosylvin conferred protection to retinal pigment epithelium cells against oxidative stress through induction of HO-1. Exposure to oxidative stress led to the development of age-related macular degeneration and caused visual impairment and blindness. (Koskela *et al.* 2014)

The discovery of transient receptor potential (TRP) cation channels, opened new possibilities also to treat inflammation and inflammatory pain. Its subfamilies, transient receptor potential ankyrin (TRPA) and transient receptor potential vanilloid (TRPV), are critical entities for the detection and modulation of pain sensations (Sousa-Valente *et al.* 2014). Ly et al. found that monomethylpinosylvin inhibited capsaicin induced pain behavior i.e. it reduced the number of paw flinches in the rat and more interestingly, resveratrol did not have this effect. Capsaicin is a known activator of TRPV1 (Yu *et al.* 2013). Pinosylvin has been shown to suppress TRPA1-mediated ion currents *in vitro* and TRPA1-mediated acute paw inflammation in mice (Moilanen *et al.* 2016).

4.1.2.2 Anti-apoptotic and chemopreventive actions

In 1986, the first identified bioactivity of pinosylvin was its antineoplastic property. Pinosylvin had a significant growth inhibitory action on human lymphoblastoid cells. (Skinnider & Stoessl 1986)

Ten years later, pinosylvin was involved in a wood-derived estrogen study where it showed only slight estrogen activity in breast cancer cells while another stilbene, isorhapontin, was highly estrogenic (Mellanen *et al.* 1996). Pinosylvin achieved a significant inhibition of cell proliferation in a concentration- and time-dependent manner in colon cancer cells and inhibited phosphorylation and activation of phosphoinositide-dependent kinase-1 (PDK1), and its downstream effector Akt. More interestingly, pinosylvin induced nonsteroidal anti-inflammatory drug-activated gene (NAG-1) expression in human colon cancer cells which was related to the suppression of tumor growth. Various compounds including naturally occurring resveratrol, PPARγ agonists as well as NSAIDs have been shown previously to induce NAG-1 (Park *et al.* 2013).

It has been proposed that pinosylvin inhibits apoptotic activity via caspase-3 in endothelial cells and promotes eNOS and Akt phosphorylation and could be utilized as a cardio-protective agent (Jeong *et al.* 2013). Pinosylvin inhibited caspase-3 activity also in human neutrophils (Perecko *et al.* 2012). It has also been reported that

pinosylvin induces cell death in bovine aortic endothelial cells at high concentrations while being anti-proliferative at low concentrations (Park *et al.* 2014).

4.2 Lignans

Lignans are plant phenols that are derived biosynthetically from phenylpropanoids (Figure 10). They occur freely in plants or co-exist with sugars. Lignans have been traditionally classified into two types, classical lignans and neolignans. Nowadays they are usually classified in five categories according to their structures: lignans, neolignans, norlignans, hybrid lignans and oligomeric lignans (Zhang *et al.* 2014). The main sources of lignans in food are cereals, grain products, vegetables, fruit, berries and beverages (Tetens *et al.* 2013).

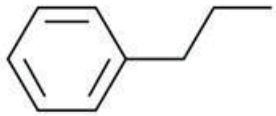


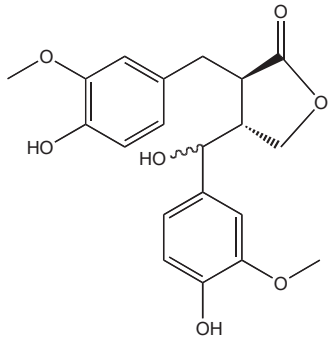
Figure 10. Basic lignan structure, the propylbenzene skeleton

Lignans have been under intensive investigation during recent years. Bioactivities like anti-inflammatory, antibacterial, cardiovascular protective, neuroprotective, anti-human immunodeficiency virus (HIV) and anti-cancer have been identified (Zhang *et al.* 2014). Etoposide, a semi-synthetic derivative of the lignan, podophyllotoxin, has been a success in cancer treatment (Kamal *et al.* 2015).

The main wood lignans found in Nordic conifers are hydroxymatairesinol, matairesinol, isolariciresinol, secoisolariciresinol, pinoresinol and nortrachelogenin. Lignans can be found in stemwood but the knots are richer sources of lignans (Holmbom *et al.* 2003). Referring to lignans the main focus of this work was on nortrachelogenin because of its existence in Scots pine knot extract and the limited amount of previous data available. Matairesinol is also a component found in the same extract but at lower concentrations and hydroxymatairesinol is a component of spruce pine knot. In addition, there is an abundance of published investigations of both components, these have tended to state that nortrachelogenin is more interesting.

4.2.1 Hydroxymatairesinol

Hydroxymatairesinol can be isolated in large quantities from the knots of Norway spruce. It belongs to the classical lignans based on the structure (Figure 11). In the last 20 years, several articles have been published about the biological activities of hydroxymatairesinol. In particular, the antitumor activities have been investigated because hydroxymatairesinol is metabolized by the microbiota into two mammalian lignans, enterodiol and enterolactone. It is a phytoestrogen like most of the lignans and structurally resembles 17-β-estradiol.



Hydroxymatairesinol

Figure 11. Chemical structure of hydroxymatairesinol

Orally administered hydroxymatairesinol has been shown to have antitumour activity in 7,12-dimethylbenz[a]anthracene (DMBA)-treated rats (Saarinen *et al.* 2001). No estrogenic, antiestrogenic, or antiandrogenic activity was detected *in vivo,* in contrast to enterolactone (Saarinen *et al.* 2000, Saarinen *et al.* 2001), although mild estrogenic activity was observed in MCF-7 human breast cancer cells *in vitro* (Cosentino *et al.* 2007). Furthermore, Saarinen et al. reported that hydroxymatairesinol had stronger anti-oxidant potency compared to enterolactone and enterodiol as measured by its capacity to inhibit tert-butyl hydroperoxide induced phospholipid oxidation in rat liver microsomes (Saarinen *et al.* 2000). This finding was supported by another study where the anti-oxidant effect was evaluated as the weight gain of C57BL/6J mice fed with alpha-tocopherol-deficient diet. The effect of hydroxymatairesinol (500 mg/kg per day) was comparable to that of DL-alpha-tocopherol (766 mg/kg per day) (Kangas *et al.* 2002). Hydroxymatairesinol or its metabolites exerted chemo-preventive properties in the rat N-ethyl-N'-nitro-N-nitrosoguanidine-uterine

carcinogenesis model and a hydroxymatairesinol diet was effective in a prostate cancer model *in vivo*, where mice treated with hydroxymatairesinol had smaller LNCaP tumors (Bylund *et al.* 2005, Katsuda *et al.* 2004).

In addition to hormone-related cancers, the effect of hydroxymatairesinol has been studied in hepatoma cells where it inhibited proliferation of AH109A cells *in vitro* and inhibited the growth and metastasis of subcutaneous AH109A hepatomas in rats (Miura *et al.* 2007).

Like other phytoestrogens, hydroxymatairesinol has been shown to have immunomodulatory activity *in vitro*. Cosentino et al. showed that hydroxymatairesinol potassium acetate inhibited LPS-induced TNF-α expression in human THP-1 monocytes. Hydroxymatairesinol was also shown to inhibit N-formylmethionyl-leucyl-phenylalanine (fMLP) and angiotensin II induced IL-8 production in polymorphonuclear leukocytes. fMLP is a chemotactic peptide on membrane receptors which directly stimulates the respiratory burst, and angiotensin II is involved in inflammation associated with hypertension and atherosclerotic plaque formation. (Cosentino *et al.* 2010)

One clinical study of hydroxymatairesinol derived from Norwegian spruce has been published. A dose comparison study was made in 22 post-menopausal females. The daily dose was 36 mg in one group and 72 mg in a second group provided for 8 weeks. Plasma hydroxymatairesinol levels increased by 191 % in low dose group and by 1238 % in the high dose group as compared to baseline. Hydroxymatairesinol reduced statistically significantly both the frequency and severity of hot flashes in post-menopausal women with no adverse events being reported. (Udani *et al.* 2013)

4.2.2 Nortrachelogenin

(+)-Nortrachelogenin, also called wikstromol, (Figure 12) was identified as a new pharmacologically active lignan in 1979 by Kato et al. when it was shown to have effects on the central nervous system by causing depression in rabbits (Kato *et al.* 1979). It was first isolated from *Wikstroemia indica*, a small shrub with red berries growing in Asia, which is one of the fundamental herbs used in Chinese medicine (Wang *et al.* 2005).

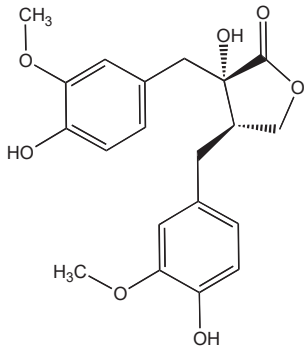


Figure 12. Chemical structure of nortrachelogenin

In 2002 (-)-nortrachelogenin was identified in the knot and branch heartwood of Scots pine (Ekman *et al.* 2002). When investigating the literature data, the names of nortrachelogenin and its enantiomer are sometimes confused and that should be taken into account because most probably stereochemistry plays a role in the activity of nortrachelogenin (Ekman *et al.* 2002, Peuhu *et al.* 2013).

Nortrachelogenin is a classical lignan structurally close to hydroxymatairesinol and matairesinol. Some plant lignans can be easily converted into mammalian lignans, enterolactone and enterodiol. Nortrachelogenin does not seem to be converted into these mammalian lignans in the gut, instead it is absorbed as such after oral administration (Saarinen *et al.* 2005). There is still rather limited data available on the pharmacological activities of nortrachelogenin, especially its anti-inflammatory properties.

4.2.2.1 Bioactivities *in vitro*

In previous studies, nortrachelogenin has been shown to have anti-plasmodial activity in the *Plasmodium falciparum in vitro* drug sensitivity test suggesting anti-malarial bioactivity (Kebenei *et al.* 2011). Its antifungal properties have also been investigated and it induced morphological deformation of *Pyriculuria oryzae* and was also moderately active against HIV-1 in an anti-HIV bioassay testing system used by the National Cancer Institute of USA (Hu *et al.* 2000). (+)-Nortrachelogenin exhibited also antitumor activity against the P-388 lymphocytic leukemia (Torrance *et al.* 1979). (−)-Nortrachelogenin isolated from *Carissa spinarum* showed cytotoxicity against breast (MCF7) and lung (A549) cancer cells (Wangteeraprasert *et al.* 2012).

In a more recent study, (-)-nortrachelogenin was reported to have anti-cancer activity in LNCaP prostate cancer cells. Peuhu et al. investigated 27 lignans or lignan derivatives, and nortrachelogenin was found to be the most effective to sensitize the androgen–deprived LNCaP prostate cancer cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a death ligand that has the ability to induce tumor-specific cell death, by inhibiting several components in RTK/PI3K/Akt pathway (Peuhu *et al.* 2013).

In previous studies (-)-nortrachelogenin isolated from *Trachelospermum jasminoides* was shown to inhibit TNFα induced NF-κB activity and had a minor effect on IL-6/STAT3 pathway but displayed no effect on the IFN-γ/STAT1 signaling pathway in stably transfected HepG2/NFκB cells (Chencheng *et al.* 2013, Liu *et al.* 2014). However, it was unclear whether the inhibition was direct or via the upregulation of other factors.

4.2.2.2 *In vivo* effects

Very limited experimental data is available in the literature on the *in vivo* effects of nortrachelogenin. The *in vivo* antileukemic properties of (+)-nortrachelogenin were documented in the early 1980s (Lee *et al.* 1981). In another study, (-)-nortrachelogenin isolated from *Pinus sylvestris* did not inhibit the growth of the DMBA-induced mammary tumors in rats but in a long-term experiment, weak endocrine-modulatory effects were seen (Saarinen *et al.* 2005).

Yatkin et al. described a study where Pine knot extract and mixture of stilbenes and lignans containing 7 % (w/w) of nortrachelogenin was effective against prostate cancer in orthotopic PC-3M-luc2 xenograft model in athymic mice. Nortrachelogenin was probably one of the active compounds in the extract / mixture explaining the *in vivo* effect based on its antiproliferative and proapoptotic effects in PC-3M-luc2 cells. (Yatkin *et al.* 2014)

4.3 Terpenes

Terpenes are produced by a variety of plants but many commercially derived terpenes are obtained from pine or other conifer resins. The main function of terpenes in plants is chemical defence against insects. Terpenoids are terpenes which have other functional groups in addition to hydrocarbons. Terpenes are formed from

five carbon C_5H_8 isoprene unit and can be classified by the number of isoprene units in the molecule. (Figure 13). (Salminen *et al.* 2008)

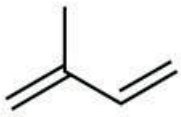


Figure 13. Isoprene unit

4.3.1 Triterpenes

Triterpenes are formed from six isoprene units. In nature, they occur as complex cyclic structures having some similarity to steroids (Figure 14). This makes them very attractive candidates for novel anti-inflammatory drugs.

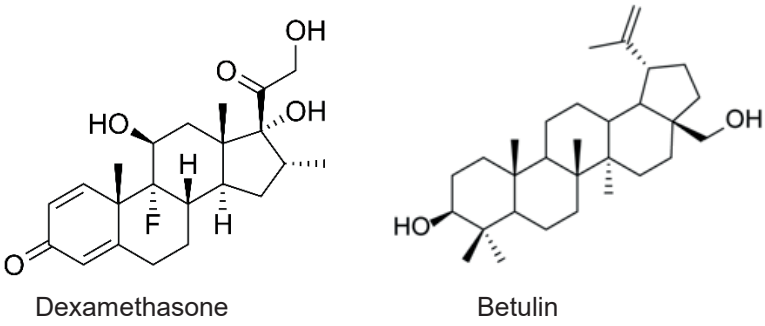


Figure 14. Chemical structures of dexamethasone and betulin

Betulin is a naturally occurring pentacyclic triterpenoid with the lupane skeleton. It is found in high amounts in the bark of the birch tree (*Betula* sp.L.). Betulin and its derivatives have a wide range of biological activities. Betulin and its semi-synthetic derivatives have been reported to have antiviral, anti-HIV, anti-malarial and anti-leishmanial properties (Alakurtti *et al.* 2010, Mukherjee *et al.* 1997, Pohjala *et al.* 2009). Betulinic acid and some of its derivatives also exhibit antitumor activity e.g. against melanoma, prostate cancer and leukemia cells (Ehrhardt *et al.* 2004, Fulda & Debatin

2005, Urban *et al.* 2004). In this study, the focus will be on the anti-inflammatory properties of betulin derivatives.

Betulinic acid has been reported to suppress LPS-induced TNF-α and IL-6 production and improved IL-10 production in mouse peritoneal macrophages (Costa *et al.* 2014). In animal studies, betulin had a significant inhibitory effect comparable to that of indomethacin in 12-O-tetradecanoylphorbol-13-acetate (TPA) ear inflammation in the mouse (Dehelean *et al.* 2012). In a carrageen induced inflammation model, betulin significantly reduced paw edema at 4 hours in the mouse (Lin *et al.* 2009). It also diminished the production of pro-inflammatory cytokines TNF-α and IL-6 and increased anti-inflammatory IL-10 levels in lung tissue in LPS induced acute lung inflammation (Wu *et al.* 2014).

Betulinic acid was also effective in carrageenan- and serotonin-induced paw edema in the rat (Mukherjee *et al.* 1997). Moreover, the effects of betulinic acid were studied in a lethal mouse model of endotoxic shock (LPS induced). A higher dose of betulinic acid (67 mg/kg) protected 100 % of animals. A reduction in TNF-α serum levels was observed but no change in IL-6 production was seen. Additionally, betulinic acid treatment increased significantly the serum levels of IL-10. Interestingly, IL-10 knock-out mice suffered greater mortality than the wild-type mice despite the betulinic acid treatment suggesting an important role of IL-10 in the immune regulation supported by betulin (Costa *et al.* 2014).

So far, only one clinical study of triterpenes has been reported. Pflugfelder et al. reported recently that the betulin-based Oleogel-S10 had no effect in the treatment of actinic keratosis. In a randomized, multicentre, placebo-controlled double-blind phase II trial, 165 patient were treated topically with Oleogel-S10 for three months. The product was well tolerated but the clinical effect did not differ from placebo (Pflugfelder *et al.* 2015).

Since there are some cyclic structural similarity in betulin and dexamethasone, we were stimulated to investigate anti-inflammatory activity of betulin and extend the studies to semi-synthetic betulin derivatives and structure-activity relationship.

Aims of the study

Trees are rich sources of biochemicals such as the stilbenoids, lignans and terpenoids; these compounds have the potential to act as immunomodulatory and anti-inflammatory compounds. In the present study, anti-inflammatory compounds were identified and their regulatory effects on the expression of inflammatory genes and inflammatory responses were investigated. Furthermore, the pharmacological mechanisms behind the effects were evaluated.

The detailed aims of this study were:

1. to study the effects of pine knot extract and stilbenoids derived from the extract on inflammatory gene expression in activated macrophages and in a standardized *in vivo* inflammation model, and to elucidate the molecular mechanisms underpinning these effects (I)

2. to investigate the possible anti-inflammatory properties of nortrachelogenin, a lignan compound derived from the pine knot extract, on inflammation *in vitro* and *in vivo* (II)

3. to screen the anti-inflammatory potential of semi-synthetic terpenoids, namely betulin derivatives by investigating their effects on the expression of inflammatory factors and to select the most promising compounds for further testing in activated macrophages and their ability to prevent an *in vivo* inflammatory response (III)

4. to investigate the involvement of IL-6 in OA and to evaluate the possible effects of pine knot extract (unpublished data) and purified stilbenoids and lignan compound nortrachelogenin (unpublished data) on the expression of IL-6 and cartilage homeostasis in human primary OA chondrocytes (IV)

Materials and methods

5 Materials

5.1 Chemicals

A knotwood extract of *Pinus sylvestris* (used in study I) and two purified compounds, pinosylvin, and monomethylpinosylvin (used in study I), were prepared in the Process Chemistry Centre, Laboratory of Wood and Paper Chemistry of Åbo Akademi University. The composition of the pine knot extract is presented in Table 9. For the studies II and IV, nortrachelogenin, pinosylvin, and monomethylpinosylvin were supplied by Oy ArboNova Ab (Turku, Finland). Betulin derivatives (used in study III) were synthesized by the Division of Pharmaceutical Chemistry and Technology, Faculty of Pharmacy, University of Helsinki. Resveratrol was purchased from Tocris Bioscience (Ellisville, MO, USA), and astringin and isorhapontin from Polyphenols Laboratoires AS (Sandnes, Norway). All test compounds were dissolved in dimethyl sulfoxide and stored at -20ºC. All other reagents were from Sigma Chemical Co (St. Louis, MO, USA).

Table 9. Composition of the Pine knot extract

Compound	Abbreviation	C [% (w/w)]	M_w (g/mol)
Monomethylpinosylvin	MePS	12	226.27
Oxidized resin acids	Ox-RA	11	~300
Pinosylvin	PS	5	212.24
Nortrachelogenin	NTG	7	374.38
Abietic acid	Ab	4	302.45
Pimaric resin acids	Pi	3	302.45
Dehydroabietic acid	DeAb	4	300.44
Palustric acid	Pal	1	302.45
Matairesinol	MR	2	358.39
TOTAL ELUTED (GC)		54	
TOTAL IDENTIFIED		49	

5.2 Antibodies

Antibodies used in western blot analysis are listed in Table 10.

Table 10. Antibodies used in western blotting analysis

Antibody type	Name	Code	Supplier
Primary antibodies	Rabbit polyclonal iNOS	sc-650	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
	Rabbit polyclonal HO-1	sc-1797	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
	Goat polyclonal COX-2	sc-1745	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
	Rabbit polyclonal mPGES1	AS03031	Agrisera AB, Vännas, Sweden
	Rabbit polyclonal β-actin	sc-1616-R	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
Secondary antibodies	Goat polyclonal anti-rabbit IgG-HRP	sc-2004	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
	Donkey polyclonal anti-goat IgG-HRP	sc-2020	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA

6 Methods

6.1 Cell cultures

6.1.1 J774 murine macrophages

Murine J774 macrophages (American Type Culture Collection, Rockville, MD, USA) were cultured at 37 °C in 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) with glutamax-I containing 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (250 ng/ml) (Invitrogen, Paisley, UK). Cells were seeded on 96-well plates for the XTT-test and on 24-well plates for the measurements of NO, MCP-1, IL-6, PGE_2, HO-1, iNOS, COX-2 and mPGES1. Cell monolayers were grown for 72 hours to confluence before the experiments were started. Cells were stimulated with LPS (10 ng/ml), IFN-γ (10 ng/ml) or cytomix (the combination of IFN-γ, IL-β and TNF-α, all 10 ng/ml) and the compounds of interest were added in fresh culture medium in 1:1000.

6.1.2 HEK293pGL4.32NFκB Cell Line

Human embryonic kidney (HEK)293 cells (ATCC, Manassas, VA, USA) were cultured at 37 °C in 5% CO_2 atmosphere in Eagle´s Minimum Essential Medium with L-glutamine containing 10% heat-inactivated fetal bovine serum and supplemented with sodium bicarbonate (0.15 %), non-essential amino acids (1mM each), sodium pyruvate (1 mM) (all from Lonza, Basel, Switzerland) and penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (250 ng/ml) (all from Invitrogen, Paisley, UK). Culture medium of transfected cells was supplemented with Hygromycin (200 μg/ml) (Thermo Fisher Scientific, Carlsbad, CA, USA) for selection.

The HEK-293 cells were stably transfected with a luciferase reporter construct, pGL4.32[luc2P/NF-κB-RE/Hygro]. The plasmid was purchased from Promega

Corp. (Madison, WI, USA) and contained five copies of an NF-κB response element that drives transcription of the luciferase reporter gene.

6.1.3 Human primary chondrocytes

Leftover pieces of OA cartilage from knee replacement surgery were used. Full-thickness pieces of articular cartilage from femoral condyles, tibial plateaus and patellar surfaces were removed aseptically from subchondral bone with a scalpel and cut into small pieces. Cartilage pieces were washed with phosphate buffered saline (PBS), and chondrocytes were isolated with enzymatic digestion for 16 hours at 37°C in a shaker by using a collagenase enzyme blend (1 mg/ml Liberase TM, Roche, Mannheim, Germany in medium). Isolated chondrocytes were washed and plated on 24-well plates (2×10^5 cells/ml) in culture medium (DMEM/U1, Lonza, Basel, Switzerland) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (250 ng/ml) containing 10% fetal bovine serum (all from Thermo Fisher Scientific, Carlsbad, CA, USA)). After 24 hours, the cells were stimulated with IL-1β (100 pg/ml) or IL-17 (50 ng/ml), both from R&D Systems Europe Ltd, Abingdon, UK and treated with the compounds of interest for times indicated.

6.1.4 T/C28a2pGL4.32NFκB Cell Line

T/C28a2 human chondrocyte cells were a kind gift from Professor Mary B. Goldring (Beth Israel Deaconess Medical Center and New England Baptist Bone & Joint Institute, Harvard Institutes of Medicine, Boston, USA). T/C28a2 cell line was cultured at 37°C in 5% CO_2 atmosphere and grown in DMEM/Ham´s F12 (Lonza) with glutamax-I containing 10 % heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (250 ng/ml) (Thermo Fisher Scientific, Carlsbad, USA).

Cells were stably transfected with luciferase reporter construct, pGL4.32[luc2P/NF-κB-RE/Hygro]. The plasmid was obtained from Promega Corporation (Madison, WI, USA) and contains five copies of an NF –κB response element that drives transcription of the luciferase reporter gene. Cells were plated on 24-well plates for the experiments.

6.1.5 Cell viability assays

Cytotoxicity of all investigated compounds was ruled out by measuring cell viability using XTT-test (Cell Proliferation Kit II, Roche Diagnostics, Mannheim, Germany) according to the manufacturer´s instructions, and by visual assessment of the cells with a microscope after the experiments in a routine manner. Viable cells metabolize sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) to formazan by mitochondrial dehydrogenase. The cells were incubated with the tested compounds and stimulant (LPS I-III and IL-1β IV) for 20 h before addition of XTT at a concentration of 0.3 mg/ml and N-methyl dibenzopyrazine methyl sulfate (1.25 mM). Cells were incubated for another 4 h and the amount of formazan accumulated into the growth medium was assessed spectrophotometrically. Triton-X treated cells were used as a positive control for cell death. Conditions were considered toxic if the cells' ability to metabolize XTT to formazan was lowered by more than 20 % as compared to cells exposed to stimulant only. None of the tested compounds were observed to cause cytotoxicity at the concentrations used.

6.2 Nitrite assays

NO production was determined by measuring the accumulation of nitrite, a stable metabolite of NO in aqueous milieu, by the Griess reaction (Green *et al.* 1982). Equal volumes (50 µl) of culture medium and Griess reagent (0.1 % naphthylethylenediamine dihydrochloride, 1 % sulfanilamine, 2.4 % H_3PO_4) were incubated together and the absorbance was measured at 540 nm. The concentration of nitrite was calculated using sodium nitrite as a standard.

6.3 Preparation of cell lysates and western blotting

At the indicated time points, cells were rapidly washed with ice-cold phosphate-buffered saline (PBS) and solubilized in cold lysis buffer containing 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 1% Triton-X-100, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 20 µg/ml leupeptin, 50 µg/ml aprotinin, 5 mM sodium fluoride, 2 mM sodium pyrophosphate and 10 µM *n*-octyl-β-D-glucopyranoside. After incubation for 15 min on ice, lysates were

centrifuged (12,000g, 4ºC for 10 min), and supernatants were collected and stored in sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl, pH 6.8, 10 % glycerol, 2 % SDS, 0,025 % bromophenol blue, and 5 % β-mercaptoethanol) in -20ºC. An aliquot of the supernatant was used to determine the protein concentration by the Coomassie blue method (Bradford 1976).

Prior to the western blot analysis, the samples were boiled for 10 min to denature the protein. Equal aliquots of protein (20 μg) were loaded on a 10 % SDS-polyacrylamide gel and separated by electrophoresis. Proteins were transferred to Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham, Buckinghamshire, U.K.) by semidry electroblotting. After transfer, the membrane was blocked in TBS/T (20 mM Trisbase pH 7.6, 150 mM NaCl, 0.1 % Tween-20) containing 5 % nonfat milk or bovine serum albumin (BSA) for 1 h at room temperature. The membrane was incubated with the primary antibody in the blocking solution overnight at 4º C, and with the secondary antibody in the blocking solution for 1 h at room temperature. Bound antibody was detected using SuperSignal West Pico or Dura chemiluminescent substrate (Pierce, Rockford, USA) and Image Quant LAS 4000 mini imaging system (GE Healthcare Bio-Sciences AB). The quantitation of the chemiluminescent signal was carried out with the use of Imaging Quant TL software (GE Healthcare Bio-Sciences AB).

6.4 RNA extraction and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

At the indicated time points, culture medium was removed and cells were washed twice with PBS, lysed and total RNA extraction was carried out with GenElute™ Mammalian Total RNA Miniprep Kit (Sigma Aldrich, St Louis, MO, USA) according to the manufacturer's instructions. The amount of RNA was measured spectrophotometrically and purity was confirmed via the absorbance ratio at A260/A280. Samples were stored at -80ºC before further use.

Total RNA was reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA). cDNA obtained from the RT-reaction was diluted 1:20 with RNAse-free water and subjected to quantitative PCR using TaqMan Universal PCR Master Mix and ABI PRISM 7000 Sequence detection system (Applied Biosystems, Foster City, CA).

Primers and probes (Table 11) for iNOS, IL-6, HO-1, aggrecan and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, used as a control gene) were

designed using Primer Express® Software (Applied Biosystems, Foster City, CA, USA) and supplied by Metabion (Martinsried, Germany). Expression of MCP-1 mRNA was measured using TaqMan® Gene Expression Assay (Applied Biosystems, Foster City, CA, USA).

The primer and probe sequences and concentrations were optimized according to the manufacturer's guidelines in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C. PCR reaction parameters were as follows: incubation at 50 °C for 2 min, incubation at 95 °C for 10 min, and thereafter 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. Each sample was determined in duplicate. A standard curve method was used to determine the relative mRNA levels as described in the Applied Biosystems User Bulletin. A standard curve for each gene was created using total RNA isolated from stimulated cells. Isolated RNA was reverse transcribed and dilution series of cDNA ranging from 1 pg to 10 ng were subjected to PCR. The obtained threshold cycle values were plotted against the dilution factor to create a standard curve. Relative mRNA levels in the test samples were then calculated from the standard curve and mRNA levels were normalized against GAPDH.

Table 11. Primer and probe sequences used in this study

Gene	Oligonucleotide	Sequence 5`→3`
Murine	Forward primer	CCTGGTACGGGCATTGCT
iNOS	Reverse primer	GCTCATGCGGCCTCCTT
	Probe	CAGCAGCGGCTCCATGACTCCC
Murine	Forward primer	TCGGAGGCTTAATTACACATGTTC
IL-6	Reverse primer	CAAGTGCATCATCGTTGTTCATAC
	Probe	CAGAATTGCCATTGCACAACTCTTTTCTCA
Murine	Forward primer	CCCTCACAGATGGCGTCACT
HO-1	Reverse primer	GCGGTGTCTGGGATGAGCTA
	Probe	CCTGCAGAGACACCCCGAGGGA
Murine	Forward primer	CCTGGATACATTTCCTCGTTGTC
mPGES-1	Reverse primer	GAAGGCGTGGGTTCAGCTT
	Probe	ACAGGCCGTGTGGTACACACCG
Murine MCP-1		TaqMan® Gene Expression Assay
Murine	Forward primer	GCATGGCCTTCCGTGTTC
GAPDH	Reverse primer	GATGTCATCATACTTGGCAGGTTT
	Probe	TCGTGGATCTGACGTGCCGCC
Human	Forward primer	TACCCCCAGGAGAAGATTCCA
IL-6	Reverse primer	CCGTCGAGGATGTACCGAATT
	Probe	CGCCCCACACAGACAGCCACTC
Human	Forward primer	GGCAATAGCAGGTTCACGTACA
Collagen II	Reverse primer	CGATAACAGTCTTGCCCCACTT
	Probe	CTGAAGGATGGCTGCACGAAACATACC
Human	Forward primer	GCCTGCGCTCCAATGACT
Aggrecan	Reverse primer	TAATGGAACACGATGCCTTTCA
	Probe	CCATGCATCACCTCGCAGCGGTA
Human	Forward primer	AAGGTCGGAGTCAACGGATTT
GAPDH	Reverse primer	GCAACAATATCCACTTTACCAGAGTTAA
	Probe	CGCCTGGTCACCAGGGCTGC

6.5 Enzyme-linked immunosorbent assay and multiplex bead array

Culture medium samples were stored at -20 °C until analyzed. IL-6 and MCP-1 concentrations were measured in the culture medium by ELISA using reagents from R&D Systems Europe Ltd (Abingdon, UK) and PGE_2 by using reagents from Cayman Chemicals (Ann Arbor, Michigan, USA). Analyses were carried out according to the manufacturer´s instructions. Detection limits were 7.8 pg/ml (IL-6), 1.95 pg/ml (MCP-1) and 7.8 pg/ml (PGE_2).

The concentration of IL-6 in plasma, synovial fluid, and culture medium was measured by ELISA with commercial reagents from Sanquin (Amsterdam, The Netherlands). The detection limit was 0.3 pg/ml. MMP-3 concentrations were assessed by ELISA (R&D Systems, Inc, Minneapolis, USA). MMP-1 concentrations in the synovial fluid were determined by multiplex bead array (Fluorokine® Human MMP Multi Analyte Profiling Base Kit, R&D systems, Minneapolis, USA). Detection limits were 10.7 pg/ml for MMP-1 and 15.6 pg/ml MMP-3.

6.6 Luciferase activity

Firefly luciferase activity was measured using the luciferase assay reagent (Promega Corp., Madison, WI, USA), and the results were normalized to the total cellular protein. The protein content was measured using the Coomassie blue method (Bradford 1976).

6.7 Carrageenan induced inflammation in the mouse

Anti-inflammatory effects were studied by measuring carrageenan-induced paw edema in 10 week old male C57BL/6 mice (Harlan Laboratories BV, Venray, Netherlands) in study I and II and in 8 week old male C57BL/6NCrl mice (Scanbur, Copenhagen, Denmark) in study III. The animal experiments were carried out in accordance with the legislation for the protection of animals used for scientific purposes (directive 2010/63/EU) and approved by the National Animal Experiment Board, approval number ESLH-2009-07700/Ym-23, granted September 23, 2009 (I and II) and approval number ESAVI/5019/04.10.03/2012, granted September 3rd, 2012 (III).

Paw edema was induced under anesthesia and all efforts were made to minimize suffering. Mice were housed under conditions of optimum light, temperature and humidity (12:12 h light-dark cycle, 22±1°C, 50-60 %) with food and water provided *ad libitum*. Mice were randomly divided into study groups: control group, compound of interest group and dexamethasone group (2 mg/kg). Doses used for studied compounds were pinosylvin (100 mg/kg), monomethylpinosylvin (100 mg/kg), nortrachelogenin (100 mg/kg) and pyrazobetulinic acid (compound 9) (10 mg/ml). Mice were dosed with 150 µl of PBS-10 % dimethyl sulfoxide (DMSO) vehicle or the tested compound by intraperitoneal injection 2 h before carrageenan was applied. The mice were anesthesized by intraperitoneal injection of 0.5 mg/kg of medetomidine (Domitor® 1 mg/ml, Orion Oyj, Espoo) and 75 mg/kg of ketamine (Ketalar® 10 mg/ml, Pfizer Oy Animal Health, Helsinki, Finland), and thereafter the mice received 30 µl (I and II) or 45 µl (III) injection in one hindpaw of saline containing λ-carrageenan 1.5 % (w/v). The contralateral paw received a corresponding volume of saline and it was used as a control. Paw volume was measured before and 3 and 6 h after carrageenan injection by plethysmometer (Ugo Basile, Comerio, Italy). Edema is expressed as the difference, in µl, between the volume changes of the carrageenan treated paw and the control paw.

6.8 Patients and clinical studies

The patients in the study (IV) fulfilled the American College of Rheumatology (ACR) classification criteria for OA (Altman *et al.* 1986). Preoperative radiographs, blood samples and synovial fluid samples were collected from 100 patients [62 females and 38 males, body mass index (BMI) 29.7 (8.3) kg/m^2, age 72 (14) years, median, interquartile range (IQR)] with OA undergoing total knee replacement surgery at Coxa Hospital for Joint Replacement, Tampere, Finland. The study was approved by the Ethics Committee of Tampere University Hospital, Finland (ethics code R06223), and carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients.

Radiographs were evaluated according to the Ahlbäck criteria, grades I to V, with grade V representing the most severe findings (Ahlbäck 1968). Blood samples were taken before the operation and synovial fluid samples were punctured aseptically in the operation room before knee replacement surgery. The plasma and synovial fluid samples were stored at -80ºC until assayed.

6.9 Statistics

Results are expressed as the mean ± standard error of mean (SEM) in the original communications I, III and IV and mean ± standard deviation (SD) in the original communication II. The statistical significance of the results was calculated by one-way ANOVA with Dunnett's post test (dose curves) or Bonferroni´s post-test (multiple comparisons) by using GraphPad InStat 3 for Windows XP (Graph-Pad Software, San Diego, CA, USA). Differences were considered significant at $*p < 0.05$, $**p<0.01$ and $***p<0.001$. EC_{50} values were calculated with GraphPad Prism version 7.01 for Windows (GraphPad Software, San Diego, CA, USA).

Clinical data were analyzed using SPSS version 17.0 for Windows software (SPSS Inc, Chicago, IL, USA). Normality of the data was analyzed by Kolmogorov-Smirnov and based on that, nonparametric tests were used. Differences between groups were tested by Wilcoxon Signed Rank Test. P-values less than 0.05 were considered significant. Pearson's r was used to analyze correlations after natural logarithm transformation so that a normal distribution could be achieved.

7 Pine knot extract and stilbenoids have anti-inflammatory and chondroprotective properties

7.1 Pine knot extract and stilbenoids inhibit NO production and iNOS, MCP-1 and IL-6 expression in mouse macrophages

The study was started by investigating the effects of pine knot extract on NO production and iNOS, MCP-1 and IL-6 expression in mouse J774 macrophages. Cells were stimulated through the TLR4 pathway by their exposure to LPS. Knot extract significantly inhibited iNOS expression and NO production as well as IL-6 and MCP-1 expression in a dose-dependent manner (Table 12 and Figures 15, 18 and 19).

In an attempt to identify the active compounds in the pine knot extract, two major stilbenoid components in the extract, pinosylvin and monomethylpinosylvin, were tested. Pinosylvin and monomethylpinosylvin as well as the control stilbenoid resveratrol were found to suppress iNOS and MCP-1 protein expression and NO production significantly and in a dose-dependent manner without major differences between the three stilbenoids. IL-6 expression was reduced similarly by monomethylpinosylvin and resveratrol but pinosylvin decreased IL-6 expression only by about 30 % at the highest concentration used (30 μM). (Table 12 and Figures 16-19)

Table 12. EC_{50} values of the inhibitory effect of pine knot extract, pinosylvin, monomethylpinosylvin and resveratrol on NO production and on iNOS, MCP-1 and IL-6 expression in LPS activated J774 macrophages

Compound	NO	iNOS	MCP-1	IL-6
Pine knot extract	3 μg/ml	3 μg/ml	11 μg/ml	26 μg/ml
Pinosylvin	13 μM	15 μM	35 μM	*
Monomethylpinosylvin	8 μM	12 μM	35 μM	13 μM
Resveratrol	6 μM	18 μM	19 μM	13 μM

*the inhibitory effect was less than 50 % at the highest concentration tested (30 μM)

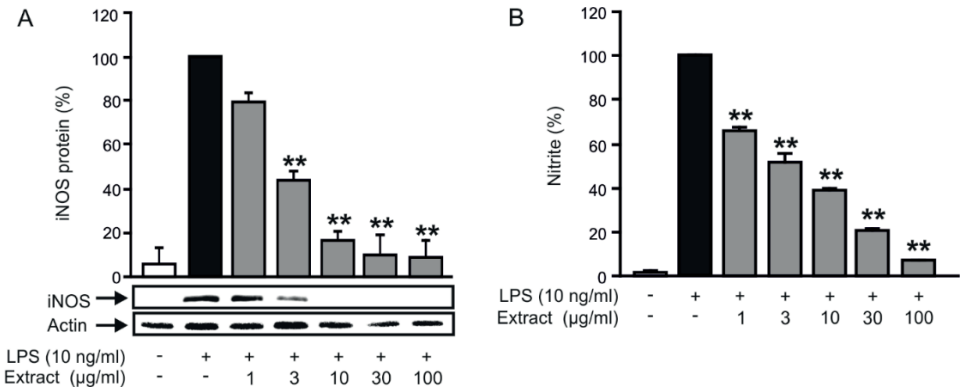


Figure 15. Effects of the *Pinus sylvestris* knot extract on LPS induced (A) iNOS protein expression and (B) NO production in J774 macrophages as measured after a 24 h incubation. iNOS expression was measured by western blot and NO production was determined by measuring its metabolite nitrite in the culture medium by the Griess reaction. Values are expressed as mean + SEM, n=4, **p<0.01 as compared to cells incubated with LPS only. (Reprinted with permission from Laavola et al. 2015 J Agric Food Chem 8;63(13):3445-53 Copyright 2015 American Chemical Society)

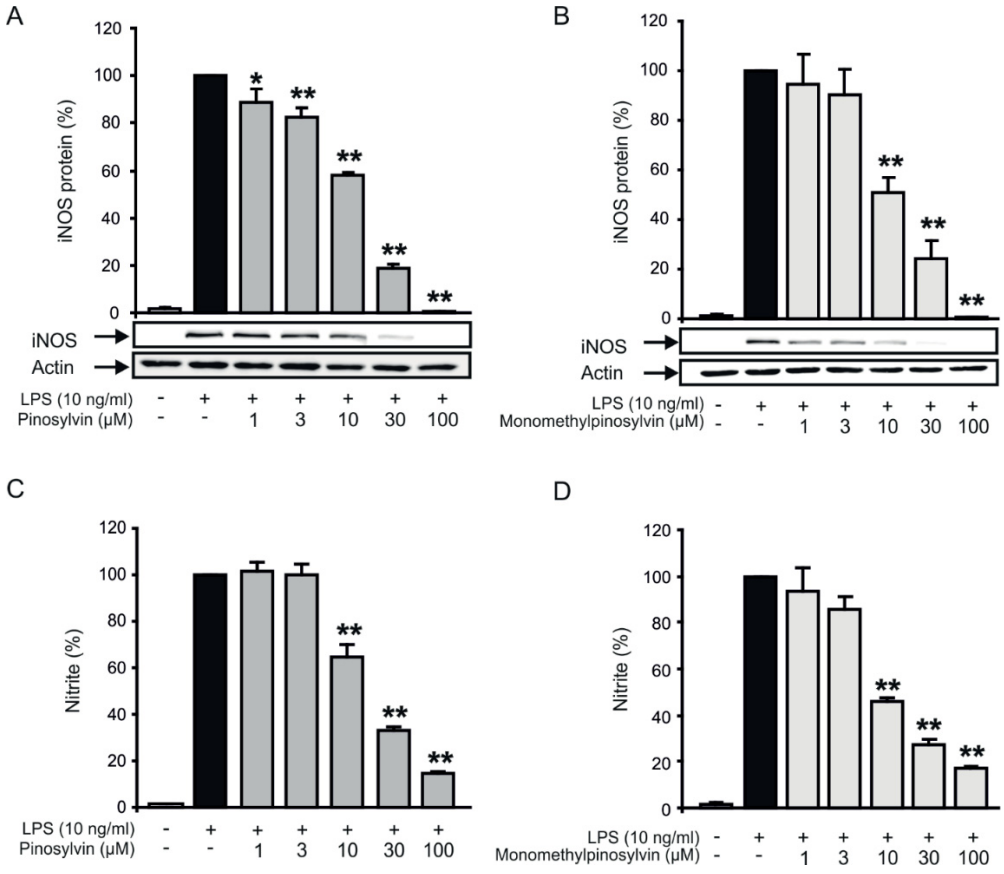


Figure 16. Effects of pinosylvin (A,C) and monomethylpinosylvin (B,D) on LPS induced (A,B) iNOS protein expression and (C,D) NO production in J774 macrophages as measured after 24 h incubation. iNOS expression was measured by western blot and NO production was determined by measuring its metabolite, nitrite, in the culture medium by the Griess reaction. Values are expressed as mean + SEM, n=4, *p<0.05 and **p<0.01 as compared to cells incubated with LPS only. (Reprinted with permission from Laavola et al. 2015 J Agric Food Chem 8;63(13):3445-53 Copyright 2015 American Chemical Society)

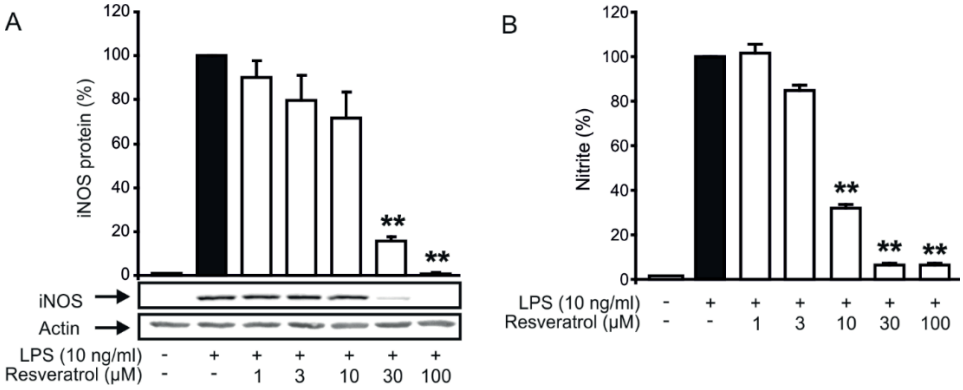


Figure 17. Effects of resveratrol on LPS induced (A) iNOS protein expression and (B) NO production in J774 macrophages as measured after 24 h incubation. iNOS expression was measured by western blot and NO production was assayed by measuring its metabolite, nitrite, in the culture medium by the Griess reaction. Values are expressed as mean + SEM, n=4, **p<0.01 as compared to cells incubated with LPS only. (Reprinted with permission from Laavola et al. 2015 J Agric Food Chem 8;63(13):3445-53 Copyright 2015 American Chemical Society)

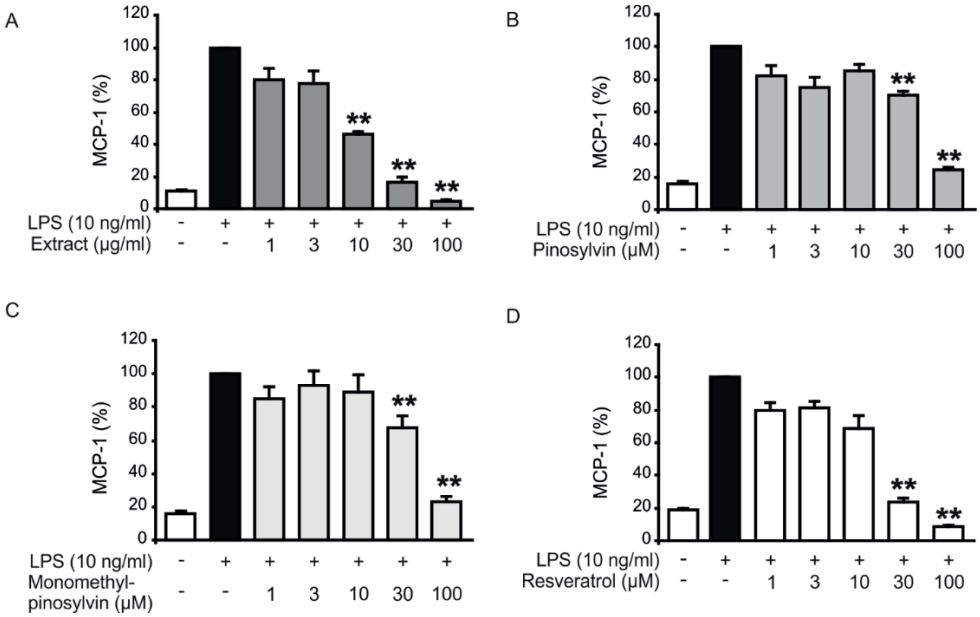


Figure 18. Effects of the *Pinus sylvestris* knot extract (A), pinosylvin (B), monomethylpinosylvin (C) and resveratrol (D) on MCP-1 production. J774 macrophages were stimulated with LPS for 24 h and thereafter the incubations were terminated and MCP-1 production was determined by ELISA. Results are expressed as mean + SEM, n=4, **p<0.01 as compared to cells incubated with LPS only. (Reprinted with permission from Laavola et al. 2015 J Agric Food Chem 8;63(13):3445-53 Copyright 2015 American Chemical Society)

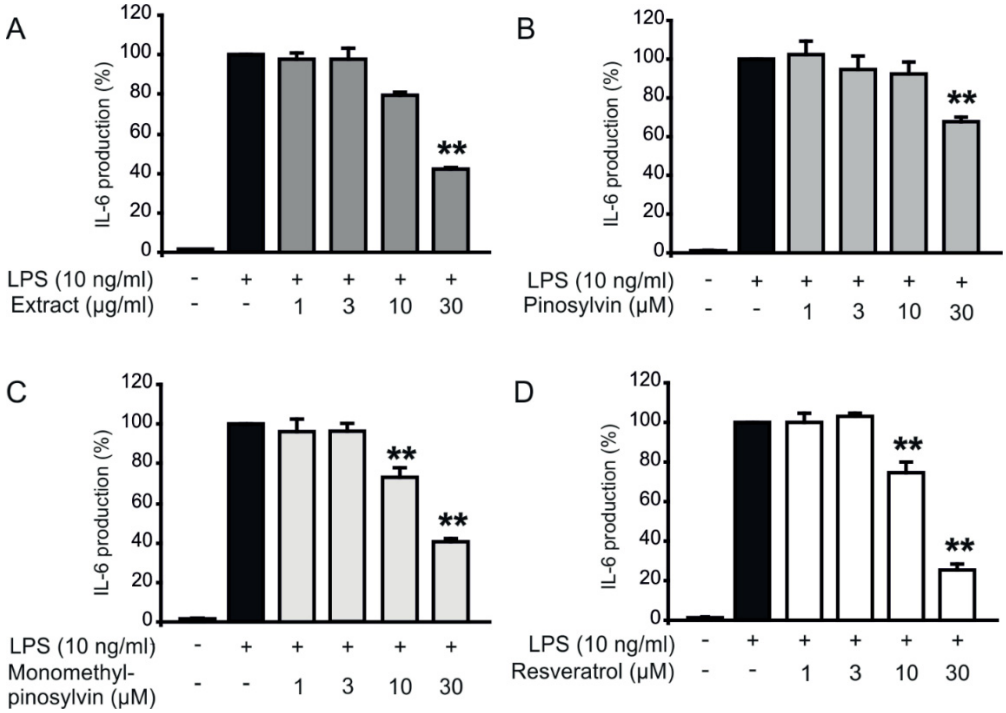


Figure 19. Effects of the *Pinus sylvestris* knot extract (A), pinosylvin (B), monomethylpinosylvin (C) and resveratrol (D) on IL-6 production. J774 macrophages were stimulated with LPS for 24 h and thereafter the incubations were terminated and IL-6 production was determined by ELISA. Results are expressed as mean + SEM, n=4, **p<0.01 as compared to cells incubated with LPS only. (Reprinted with permission from Laavola et al. 2015 J Agric Food Chem 8;63(13):3445-53 Copyright 2015 American Chemical Society)

In further studies, pine knot extract, pinosylvin, monomethylpinosylvin and the control compound resveratrol were shown to inhibit also iNOS mRNA expression in macrophages. mRNA levels were measured at the 6 h time point based on the time curve (Figure 20).

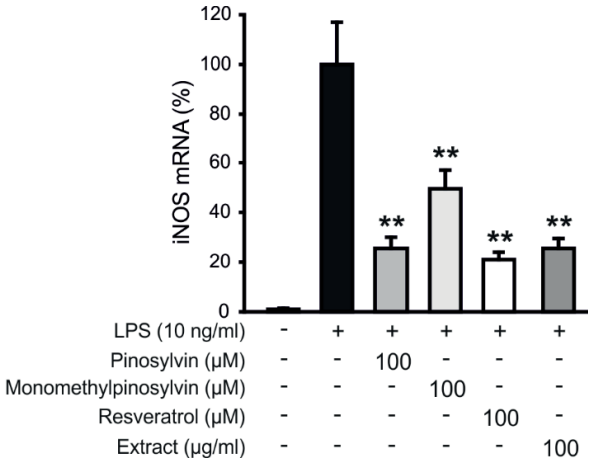


Figure 20. Effects of pinosylvin, monomethylpinosylvin, resveratrol and the *Pinus sylvestris* knot extract on iNOS mRNA expression. J774 macrophages were stimulated with LPS in the presence or in the absence of the compounds and the extract for 6 hours and iNOS mRNA levels were measured by quantitative RT-PCR. The results were normalized against GAPDH mRNA and are expressed as mean + SEM, n=4, **p<0.01 as compared to cells incubated with LPS only. (Reprinted with permission from Laavola et al. 2015 J Agric Food Chem 8;63(13):3445-53 Copyright 2015 American Chemical Society)

7.2 IL-6 levels in OA patients and chondroprotective effects of stilbene derivatives

IL-6 is a pro-inflammatory cytokine involved in the pathogenesis of OA (Wojdasiewicz *et al.* 2014). In the present study, we found that in patients with OA undergoing total knee replacement surgery (n=100), synovial fluid levels of IL-6 [119.8 (193.5) pg/ml, median (IQR)] were considerably higher than plasma levels [3.1 (2.7) pg/ml, median (IQR)]. Preoperative radiographs of the knees were evaluated by the Ahlbäck classification from grades 1 to 5. Grades 1, 2 and 3 were combined; as were grades 4 and 5. Mean synovial fluid IL-6 concentrations were higher (p=0.027) in the grades 4 and 5 group [234.1 (264.7) pg/ml, median (IQR)] than in the grade 1 to 3 groups [94.6 (183.0) pg/ml, median (IQR)]. Furthermore, synovial fluid levels of IL-6 correlated with those of MMP-1 ($r = 0.446$, $p < 0.001$) and MMP-3 ($r = 0.486$, $p < 0.001$) (Figure 21).

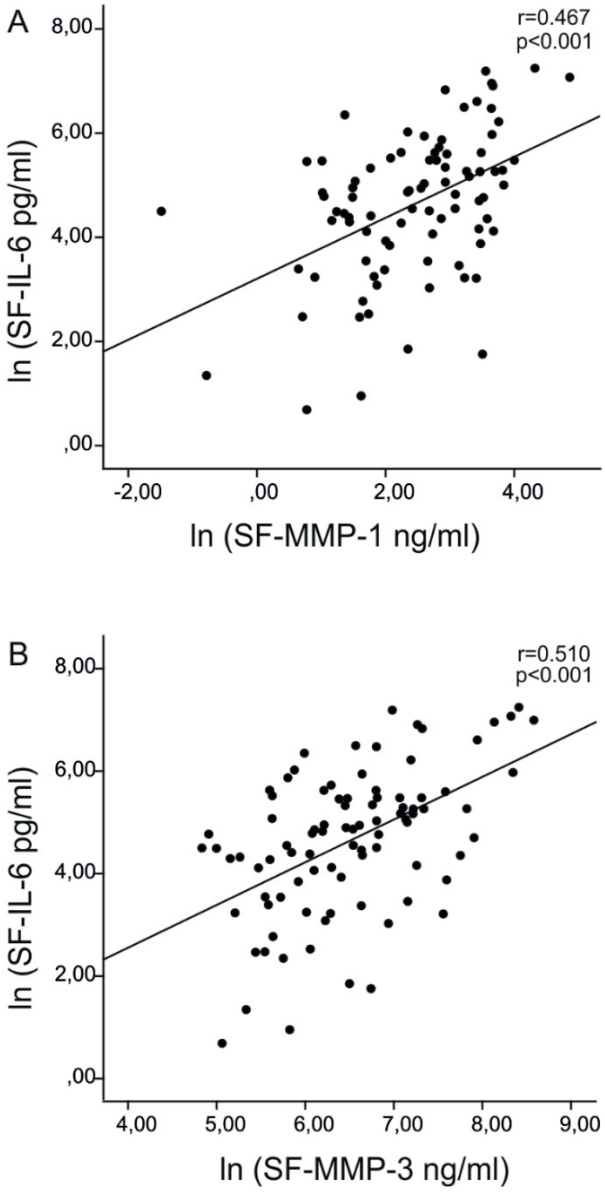


Figure 21. Correlation between IL-6 and MMP-1 (A) and MMP-3 (B) in patients with osteoarthritis (OA). IL-6 and MMPs were measured in synovial fluid (SF) by immunoassay. Natural logarithms (LN) were formed of the SF levels of IL-6 and MMPs in order to have normally distributed variables for the Pearson correlation analysis. Correlation coefficients (r) and p values are indicated. Samples were collected from 100 OA patients [BMI 29.7 (8.3) kg/m2, age 72 (14) years, median (IQR); 62/38 females/males]. (Reprinted with permission from Laavola et al. 2018 Molecules. 24(1) doi: 10.3390/molecules24010109. Licensed under CC BY 4.0)

Because IL-6 is an important factor in OA, we wanted to study if stilbene derivatives, which had previously been shown to possess anti-inflammatory properties, could also exert chondroprotective effects. Human primary chondrocytes were stimulated with two cytokines, IL-1β or IL-17, both of which have been implicated in the pathogenesis of OA (Kapoor *et al.* 2011). Based on the time curves of the effects of IL-1β and IL-17, the 24 h time point was selected for subsequent experiments.

IL-6 production and IL-6 mRNA expression were inhibited by the pine knot extract, pinosylvin, monomethylpinosylvin, resveratrol and, as anticipated, by the known anti-inflammatory compound dexamethasone similarly with both stimulants in human primary OA chondrocytes. No inhibitory effect was seen with the lignan compound nortrachelogenin, also a component of the pine knot extract. (Figure 22)

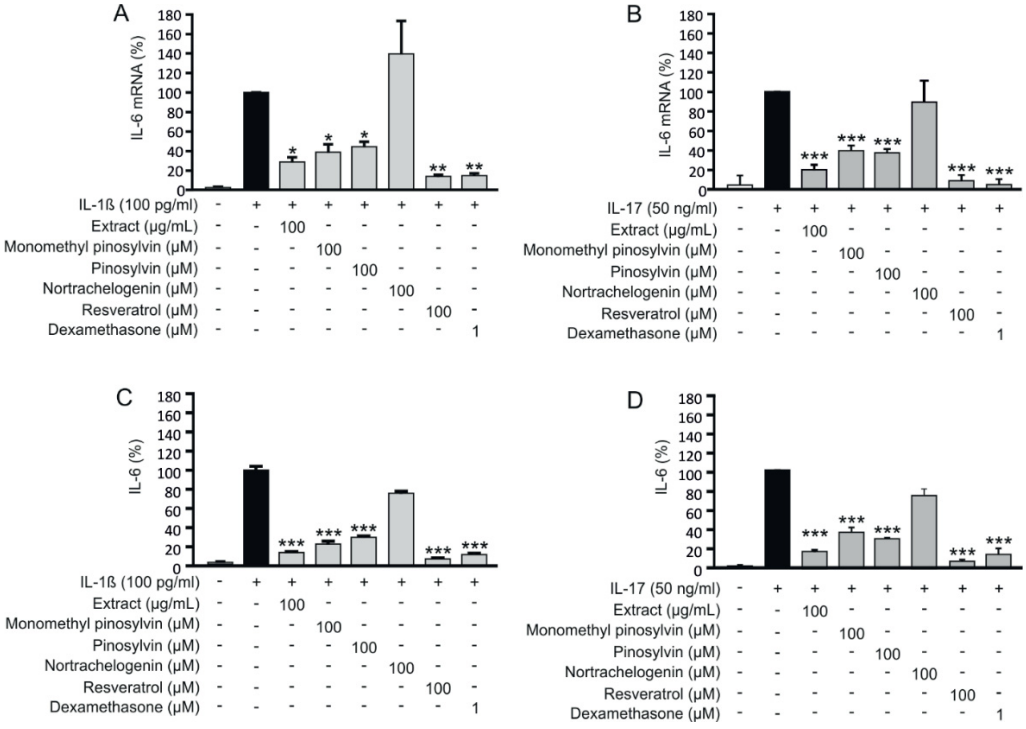


Figure 22. Effects of Pine knot extract, monomethylpinosylvin, pinosylvin, nortrachelogenin, resveratrol and control compound dexamethasone in IL-1β and IL-17 stimulated human primary chondrocytes on IL-6 mRNA expression (A and B) and IL-6 production (C and D) at time point 24h. IL-6 mRNA expression was measured by quantitative RT-PCR and results were normalized against GAPDH mRNA. IL-6 concentrations in the culture media were determined by immunoassay. IL-6 levels were 12.49-25.77 ng/ml in IL-1β and 2.60-10.22 ng/ml in IL-17 stimulated cells. Primary chondrocyte samples were obtained from three different donors and the experiments were performed in duplicate. Results are expressed as mean + SEM. * = p<0.05, ** = p<0.01 and *** = p<0.001 as compared to cells treated with IL-1β or IL-17 only.

Aggrecan and collagen II are both major components of cartilage extracellular matrix and their production is reduced in OA cartilage (Troeberg & Nagase 2012). Next, we showed that pine knot extract, pinosylvin, monomethylpinosylvin, resveratrol and, as expected, the positive control compound, dexamethasone, increased the expression of aggrecan mRNA in non-stimulated, as well as in IL-1β and IL-17 stimulated human primary chondrocytes but had no effect on the expression of collagen II. The lignan component, nortrachelogenin, had no effect on the expressions of aggrecan or collagen II. (Figure 23)

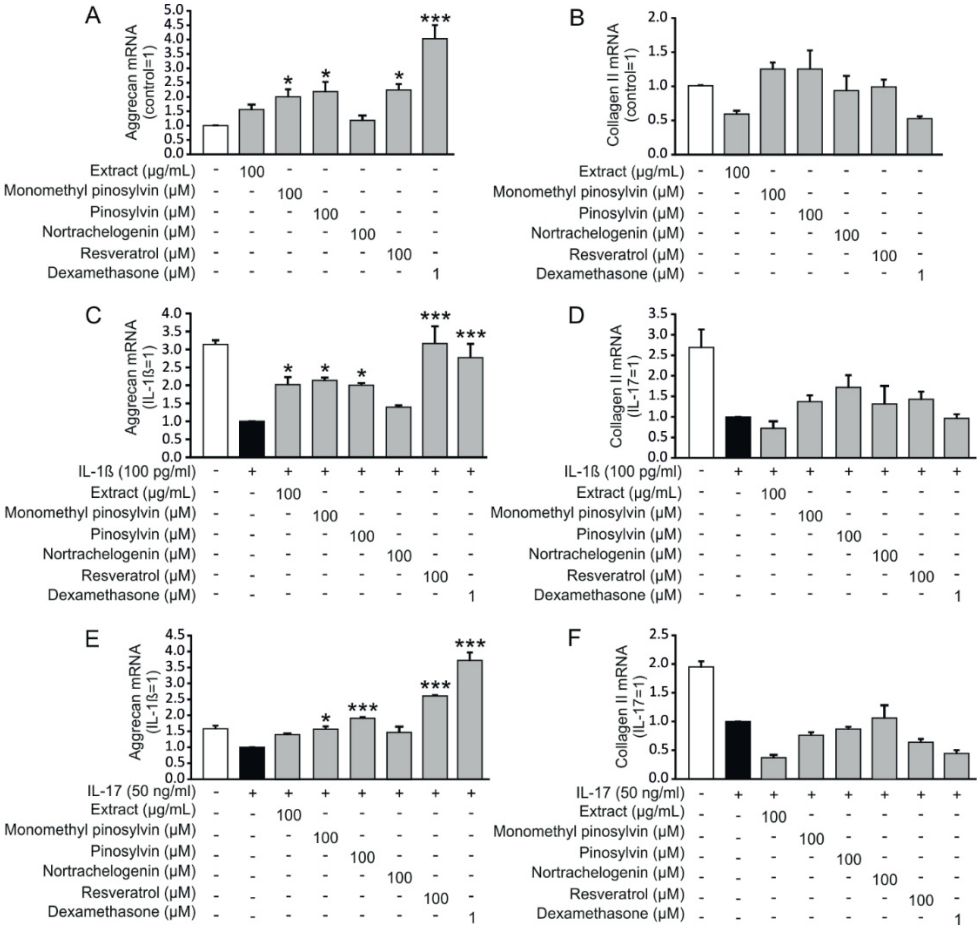


Figure 23. Effects of Pine knot extract, pinosylvin, monomethylpinosylvin, nortrachelogenin, resveratrol, a known anti-inflammatory stilbenoid and the control compound dexamethasone on aggrecan and collagen II mRNA levels. Human primary chondrocytes were cultured with the tested compounds alone (A and B) or with IL-1β (C and D) or IL-17 (E and F) for 24 h and thereafter total RNA was extracted. mRNA expression was measured by quantitative RT-PCR and the results were normalized against GAPDH mRNA. Primary chondrocyte samples were obtained from three different donors and the experiments were performed in duplicate. Results are expressed as mean + SEM. * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$ as compared to non-stimulated cells or to cells treated with IL-1β or IL-17 only.

7.3 Pine knot extract and stilbenoids inhibit NF-κB mediated transcription and increase HO-1 expression

NF- κB is an important transcription factor regulating the iNOS and IL-6 genes. Pinus sylvestris extract, pinosylvin, monomethylpinosylvin and resveratrol significantly inhibited NF-κB mediated transcription; this was investigated in the HEK293pGL4.32NFκB cell line engineered to express the reporter gene luciferase (LUC) under the control of NF-κB responsible promoter (Figure 24). MG-132, a known NF-κB inhibitor, which was included in the experiments as a positive control, decreased LUC-activity by more than 90 %.

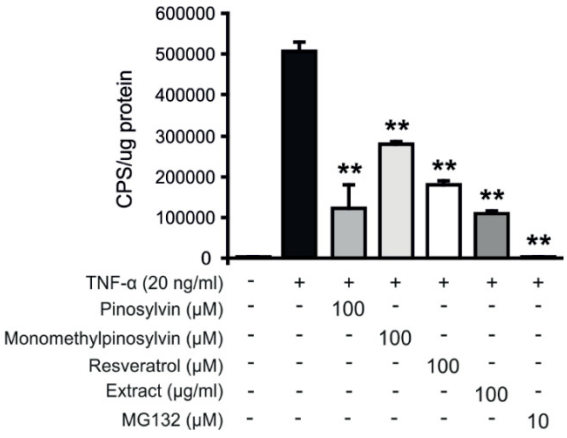


Figure 24. Effects of pinosylvin, monomethylpinosylvin, resveratrol and the *Pinus sylvestris* knot extract on NF-kB-dependent transcription in HEK-293 cells transfected with luciferase reporter construct. HEK293pGL4.32NFκB cells were stimulated with TNFα and treated with the extract or the compounds of interest or with the known NF-κB inhibitor MG132 for 5h and luciferase activity was measured. Results are expressed as mean + SEM, n=4, ** $p < 0.01$ as compared to cells incubated with TNFα only. (Reprinted with permission from Laavola et al. 2015 J Agric Food Chem 8;63(13):3445-53 Copyright 2015 American Chemical Society)

Accordingly, NF-κB mediated transcription was significantly inhibited also in the T/C28a2pGL4.32NFκB human chondrocyte cell line (measured as luciferase activity) by the extract and stilbenoid compounds but not by nortrachelogenin (Figure 25).

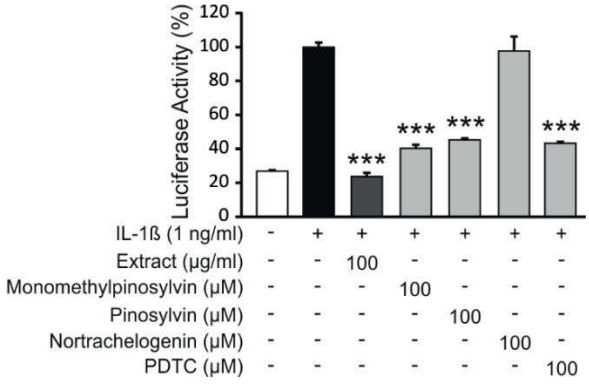


Figure 25. Effects of *Pinus sylvestris* knot extract, pinosylvin, monomethylpinosylvin and nortrachelogenin on NF-kB-mediated transcription in T/C28a2 human chondrocyte cells transfected with luciferase reporter construct. T/C28a2pGL4.32NFκB cells were stimulated with IL-1β and treated with the extract or the compounds of interest or with the known NF-κB inhibitor PDTC for 5h and luciferase activity was measured. Results are expressed as mean + SEM, n=4, ** $p < 0.01$ as compared to cells incubated with IL-1β only. (Reprinted with permission from Laavola et al. 2018 Molecules. 24(1) doi: 10.3390/molecules24010109. Licensed under CC BY 4.0)

Heme oxygenase 1 (HO-1) is a factor known to regulate NF-κB activation in macrophages. The knotwood extract, pinosylvin and monomethylpinosylvin, as well as hemin, a HO-1 inducer used as a positive control, all enhanced the HO-1 mRNA and protein levels in macrophages exposed to LPS (Figure 26).

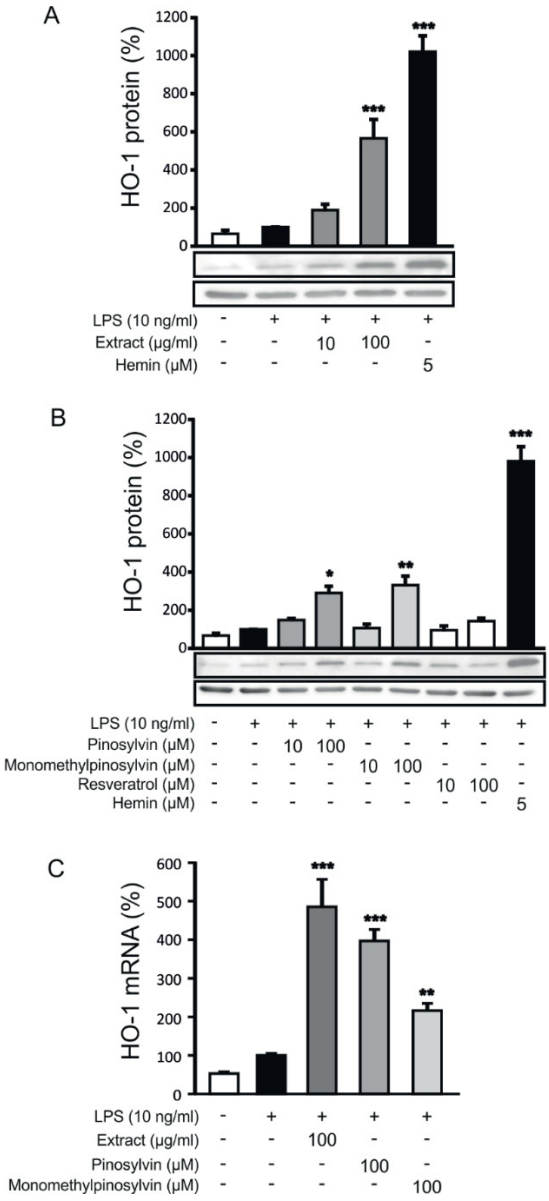


Figure 26. Effects of pinosylvin, monomethylpinosylvin, resveratrol and the *Pinus sylvestris* knot extract on HO-1 protein expression (A and B) and mRNA expression (C). J774 macrophages were stimulated with LPS in the presence or in the absence of the compounds or the extract for 6 hours and HO-1 protein expression was measured by western Blot (A and B) or for 4 hours and HO-1 mRNA expression was measured by quantitative RT-PCR (C). In (C) the results were normalised against GAPDH mRNA. The values are expressed as mean + SEM, n=4, **p<0.01 as compared to cells incubated with LPS only. (Reprinted with permission from Laavola et al. 2015 J Agric Food Chem 8;63(13):3445-53 Copyright 2015 American Chemical Society)

7.4 Stilbenes inhibit acute inflammation *in vivo*

Since both pinosylvin and monomethylpinosylvin were shown to possess anti-inflammatory effects in the *in vitro* studies, we wanted to test if those could be observed also *in vivo* in the carrageenan-induced mouse paw inflammation model. Intraperitoneal administration of previously known anti-inflammatory compounds, iNOS inhibitor L-NIL (50 mg/kg) and the glucocorticoid dexamethasone (2 mg/kg) reduced carrageenan-induced paw edema by over 80 %. Interestingly, also pinosylvin (100 mg/kg) and monomethylpinosylvin (100 mg/kg) suppressed carrageenan-induced inflammation by almost 80 % whereas the inhibitory effect of resveratrol (100 mg/kg; used as a control compound) was milder, being about 50 % (Figure 27).

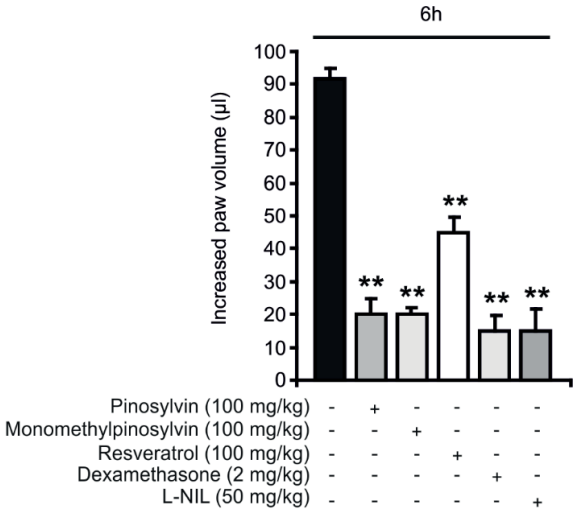


Figure 27. Effects of pinosylvin, monomethylpinosylvin and resveratrol on carrageenan-induced paw inflammation in the mouse. Pinosylvin, monomethylpinosylvin, resveratrol and the control compounds L-NIL and dexamethasone were administered i.p. 2 h prior to carrageenan (1.5 %) was injected into the paw; the contralateral paw was injected with the solvent and served as the control. Paw edema was measured before and 6 h after the carrageenan injection. Edema is expressed as the difference in the volume change between the carrageenan treated paw and the control paw. Results are expressed as mean + SEM, n=6, **p<0.01. (Reprinted with permission from Laavola et al. 2015 J Agric Food Chem 8;63(13):3445-53 Copyright 2015 American Chemical Society)

8 Nortrachelogenin is anti-inflammatory *in vitro* and *in vivo*

8.1 Anti-inflammatory properties of nortrachelogenin in activated macrophages

Nortrachelogenin is a lignan compound found in the pine knot extract (7 % w/w), in the extract used in the present study. Nortrachelogenin decreased iNOS protein expression and NO production in a dose-dependent manner in LPS stimulated J774 macrophages (Figure 28). The effect was not stimulus-specific as nortrachelogenin (30 μM) also inhibited the NO production induced by IFN-γ or by the combination of IFN-γ, IL-β and TNF-α (cytomix in Figure 28).

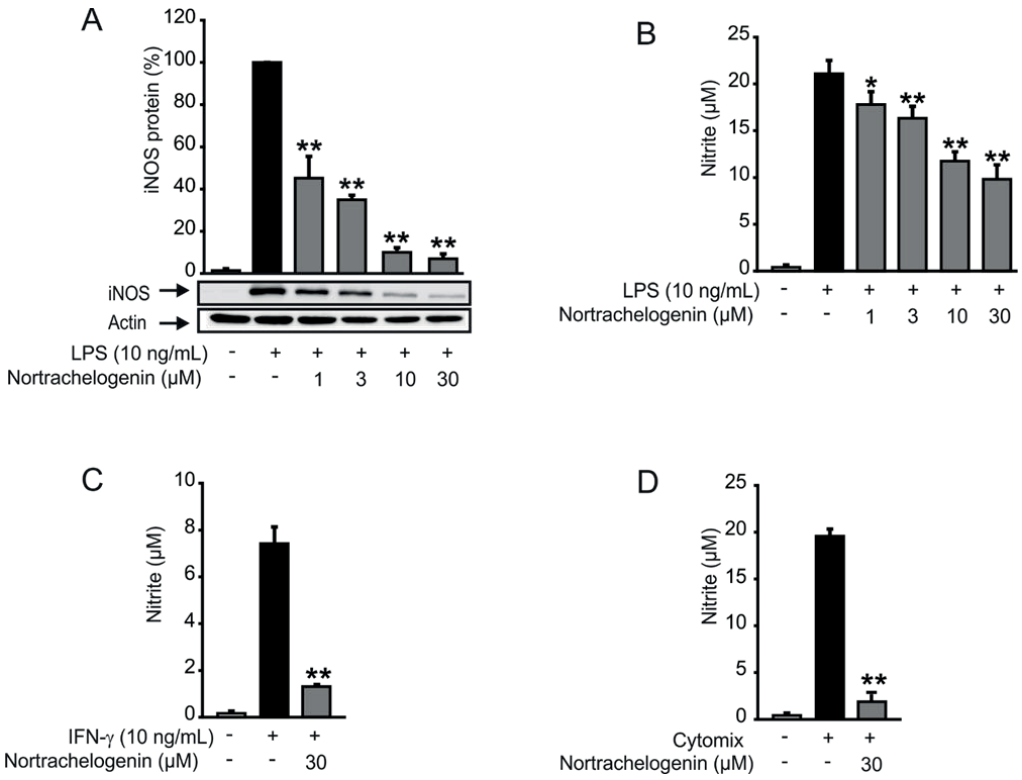


Figure 28. Effects of nortrachelogenin on LPS induced iNOS protein expression (A) and LPS, IFN-γ and cytomix (combination of IFN-γ, IL-β and TNF-α, all 10 ng/ml) induced NO production (B and C) in J774 macrophages. iNOS expression was measured by western blot and NO production as its stable metabolite nitrite by the Griess reaction after 24 h incubation. Values are expressed as mean + SD, n=4, *p<0.05 and **p<0.01 as compared to cells stimulated in the absence of nortrachelogenin. (Reprinted with permission from Laavola et al. 2017 Planta Med 83(6):519-526 Copyright 2017 Georg Thieme Verlag KG Stuttgart New York)

Nortrachelogenin had no effect on iNOS mRNA levels at any time point measured (Figure 29). The maximal iNOS mRNA levels were found following 6 h incubation with LPS, and thereafter the mRNA levels decreased rapidly.

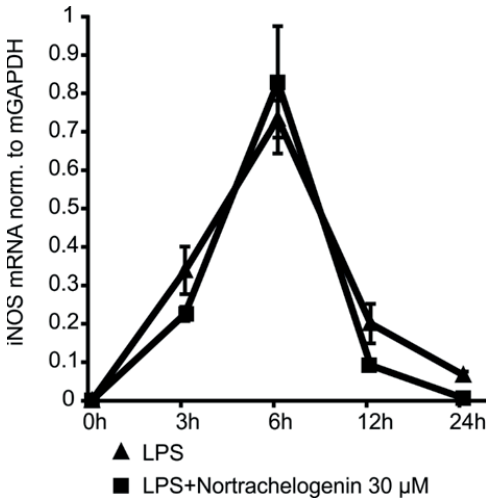


Figure 29. Effects of nortrachelogenin on LPS induced iNOS mRNA expression in J774 macrophages. RNA was extracted at time points 3h, 6h, 12h and 24h, iNOS mRNA expression was measured by RT-PCR. The results were normalised against GAPDH mRNA. Values are expressed as mean + SD, n=4, *p<0.05 and **p<0.01 as compared to cells cultured with LPS only. (Reprinted with permission from Laavola et al. 2017 Planta Med 83(6):519-526 Copyright 2017 Georg Thieme Verlag KG Stuttgart New York)

Nortrachelogenin did not reduce iNOS mRNA levels and the inhibitory effect on NO production was smaller than that on iNOS protein expression. Therefore, we postulated that nortrachelogenin might decrease iNOS protein level by enhancing iNOS protein degradation. Furthermore, there was previous data showing that iNOS is degraded by the proteasome pathway and that some pharmacological compounds enhance that effect (Paukkeri *et al.* 2007). Therefore, we examined the effect of nortrachelogenin on LPS-induced iNOS expression in the presence of the proteasome inhibitor, lactacystin (Figure 30). Interestingly, the result was in accord with the hypothesis and the presence of lactacystin blocked the inhibitory effect of nortrachelogenin on iNOS expression.

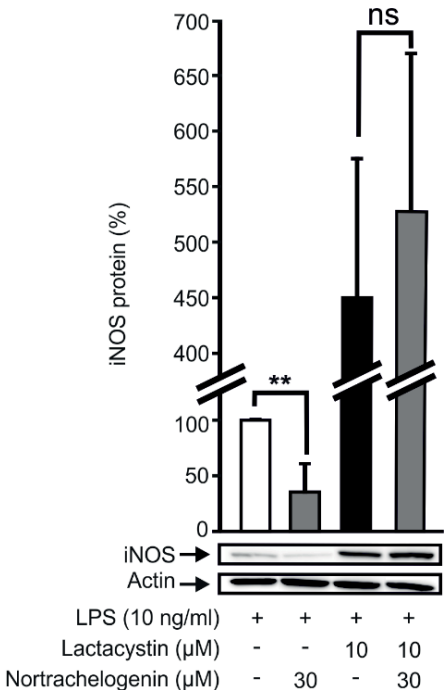


Figure 30. Effects of the proteasome inhibitor lactacystin and nortrachelogenin on iNOS expression in J774 macrophages. Cells were stimulated with LPS in the presence and in the absence of nortrachelogenin. After an 8 h incubation, lactacystin was added into the culture. Proteins were extracted after 24 h incubation and iNOS protein levels were measured by western blot. Values are expressed as mean + SEM, n=4, **p<0.01 as compared to cells cultured with LPS only. (Reprinted with permission from Laavola et al. 2017 Planta Med 83(6):519-526 Copyright 2017 Georg Thieme Verlag KG Stuttgart New York)

Nortrachelogenin reduced also MCP-1 and IL-6 expression, two other important inflammatory genes, in a dose-dependent manner (Figure 31).

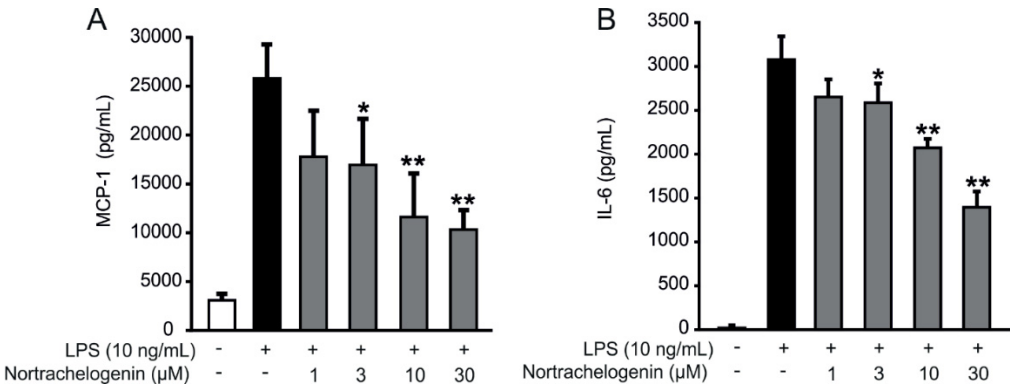


Figure 31. Effects of nortrachelogenin on MCP-1 (A) and IL-6 (B) production. J774 macrophages were stimulated with LPS in the presence of increasing concentrations of nortrachelogenin for 24 h before the incubations were terminated and MCP-1 and IL-6 concentrations in the culture media were determined by ELISA. Results are expressed as mean + SEM, n=4, *p<0.05 and **p<0.01 as compared to cells cultured with LPS only. (Reprinted with permission from Laavola et al. 2017 Planta Med 83(6):519-526 Copyright 2017 Georg Thieme Verlag KG Stuttgart New York)

PGE_2 is another important inflammatory mediator, which has a key role in inflammation, causing swelling, fever and pain (Kawahara *et al.* 2015). Interestingly, nortrachelogenin repressed PGE_2 release in macrophages in a dose dependent manner, but had no effect on COX-2 expression. Therefore, we investigated the possibility that nortrachelogenin could inhibit PGE_2 formation by decreasing the expression of mPGES-1, an inducible downstream enzyme involved in PGE_2 production. Nortrachelogenin reduced mPGES-1 expression at a concentration of 30 μM. (Figure 32)

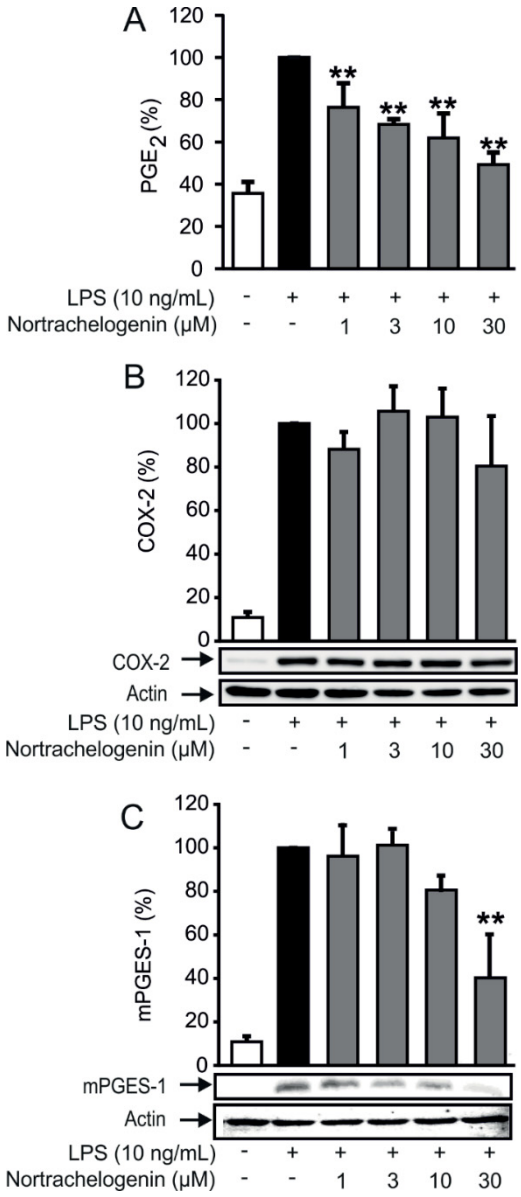


Figure 32. Effects of nortrachelogenin on LPS-induced PGE_2 production (A), COX-2 protein expression (B) and mPGES-1 protein expression (C) were measured in J774 macrophages after 24 h incubation. COX-2 and mPGES-1 protein levels were measured by western blot and PGE_2 production by ELISA. Values are expressed as mean + SD, n=4, **p<0.01 as compared to cells cultured with LPS only. (Reprinted with permission from Laavola et al. 2017 Planta Med 83(6):519-526 Copyright 2017 Georg Thieme Verlag KG Stuttgart New York)

8.2 Nortrachelogenin reduces acute inflammatory response in the mouse

While nortrachelogenin proved to have significant anti-inflammatory effects *in vitro*, we wanted to test if those effects could also be observed *in vivo*. Intraperitoneal administration of nortrachelogenin (100 mg/kg) reduced carrageenan-induced paw inflammation in the mouse significantly at 3 and 6 hours as did also the positive control compound, dexamethasone (Figure 33).

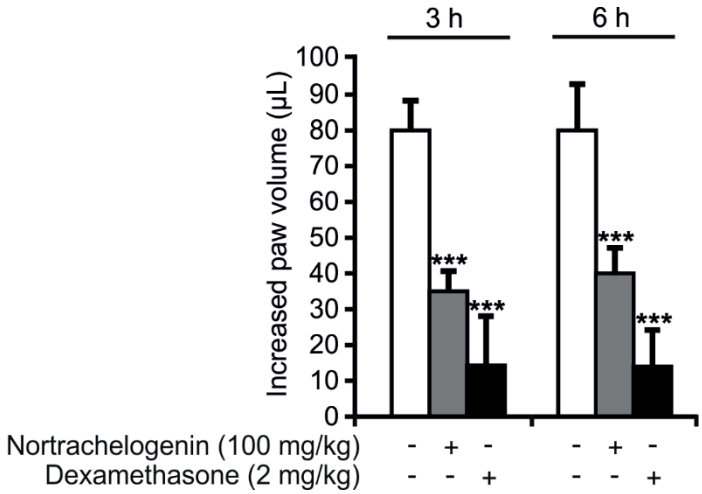
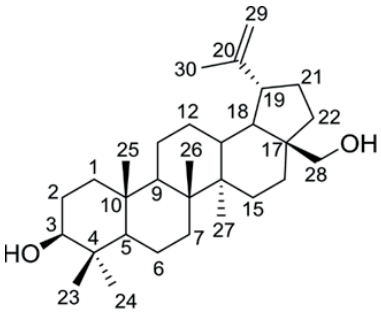


Figure 33. Effects of nortrachelogenin and the anti-inflammatory glucocorticoid dexamethasone on carrageenan-induced paw inflammation model in the mouse. Nortrachelogenin (100 mg/kg) and dexamethasone (2 mg/kg) were administered i.p. 2 h prior to carrageenan (1.5 %) was injected into the paw. Paw edema was measured before, 3 h and 6 h after carrageenan injection by a plethysmometer. Edema is expressed as the difference in volume changes between the carrageenan treated paw and the contralateral saline-injected paw. Results are expressed as mean + SEM, n=6, ***p<0.001 as compared to mice without drug treatment. (Reprinted with permission from Laavola et al. 2017 Planta Med 83(6):519-526 Copyright 2017 Georg Thieme Verlag KG Stuttgart New York)

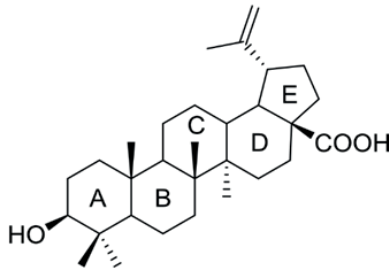
9 Immunomodulatory properties of semi-synthetic betulin derivatives

9.1 Effects of betulin derivatives on NO production and iNOS, COX-2, IL-6 and MCP-1 expression in macrophages

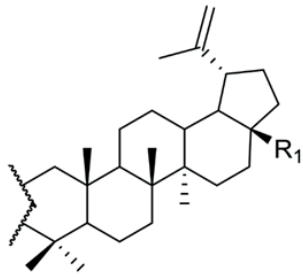
Betulin is a major component of the bark of the birch tree; it has been shown to have anti-inflammatory properties (Costa *et al.* 2014, Dehelean *et al.* 2012, Wu *et al.* 2014). It was hypothesized that by modifying betulin chemically it could be possible to increase its anti-inflammatory activity. A total of sixteen betulin derivatives together with betulin (**1**) and betulinic acid (**2**) were screened in J774 mouse macrophages for their anti-inflammatory properties (Figure 34 and 35).



Betulin (**1**)



Betulinic acid (**2**)



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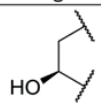
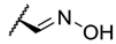
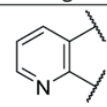
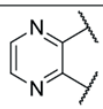
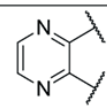
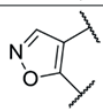
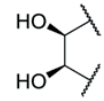
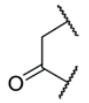
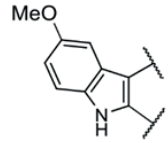
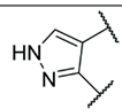
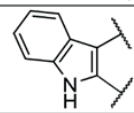
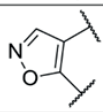
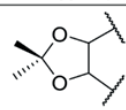
3			**11**		COOH
4		COOH	**12**		$CONH_2$
5		$CONH_2$	**13**		COOH
6		COOH	**14**		COOH
9		COOH	**17**		COOH
10			**18**		COOH

Figure 34. Structures of betulin and betulinic acid derivatives

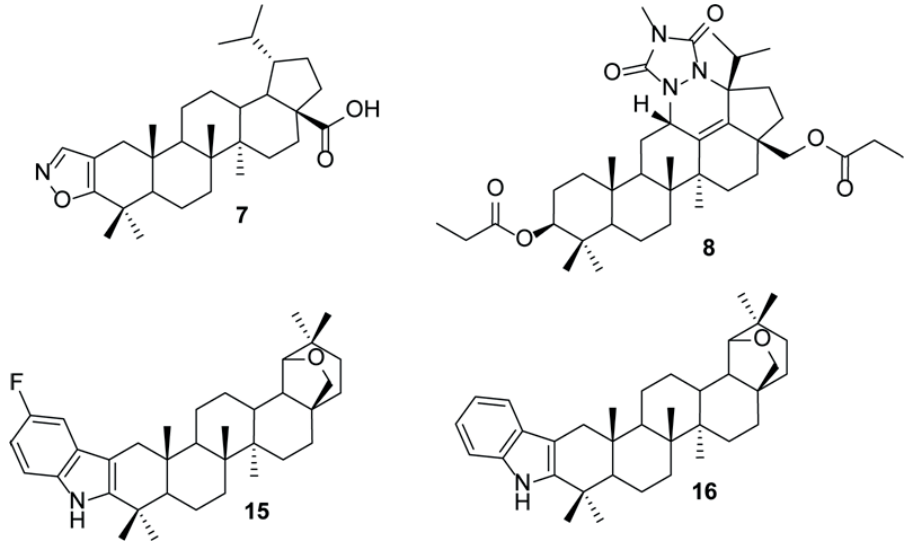


Figure 35. Structures of compounds (**7**), (**8**), (**15**) and (**16**)

Fifteen out of eighteen compounds inhibited significantly iNOS protein expression. Nine compounds caused over 50 % inhibition of iNOS protein expression at 10 μM concentration and three compounds, i.e. (**3**), (**4**) and (**5**) reduced iNOS protein expression by more than 90% an inhibitory effect comparable to that of the highly effective anti-inflammatory agent, the glucocorticoid dexamethasone. Thirteen of the tested compounds also inhibited NO production in a statistically significant manner. Interestingly, three of the compounds (**8-10**) reduced COX-2 protein expression in a statistically significant manner with compound (**8**) being the most potent at reducing COX-2 by 57 % whereas about 90% down-regulation was achieved with the known anti-inflammatory compound dexamethasone (10 μM).

Five compounds decreased IL-6 production in a statistically significant manner. A substantial reduction, over 60%, was seen only with compound (**9**) which was also the most effective compound inhibiting the MCP-1 production (over 70%) in activated macrophages. A total of five compounds suppressed MCP-1 production in a statistically significant manner. The reduction of the control compound dexamethasone (10 μM) was 38 % on IL-6 and 86 % on MCP-1. (Table 13)

.

Table 13. Effects of betulin derivatives on NO production, and iNOS, COX-2, IL-6 and MCP-1 expression in J774 macrophages. Values are expressed as mean ± SEM, n=4, * = p<0.05, ** = p<0.01 and *** = p<0.001 as compared to cells cultured with LPS only.

Betulin derivative (10 µM)	NO (mean ± SEM, %)	iNOS (mean ± SEM, %)	COX-2 (mean ± SEM, %)	IL-6 (mean ± SEM, %)	MCP-1 (mean ± SEM, %)
LPS control	100	100	100	100	100
1	47.9 ± 4.6 ***	63.4 ± 5.3 ***	175.1 ± 18.0	133.9 ± 5.6	206.5 ± 6.5
2	69.0 ± 0.8 ***	57.6 ± 5.8 ***	96.0 ± 7.8	66.1 ± 5.4 ***	98.5 ± 5.1
3	31.3 ± 2.3 ***	6.2 ± 1.0 ***	127.0 ± 4.5	98.3 ± 2.3	136.9 ± 5.2
4	41.7 ± 2.9 ***	8.3 ± 0.5 ***	144.1 ± 14.3	100.1 ± 5.0	138.4 ± 4.5
5	66.5 ± 4.2 ***	8.3 ± 1.0 ***	105.5 ± 4.8	90.9 ± 6.2	57.6 ± 2.0 ***
6	45.3 ± 1.6 ***	38.3 ± 4.7 ***	107.1 ± 8.3	87.1 ± 2.5	118.0 ± 4.7
7	40.5 ± 2.4 ***	14.5 ± 4.0 ***	88.5 ± 10.4	69.2 ± 3.0 **	65.3 ± 3.6 **
8	55.7 ± 8.2 ***	50.5 ± 4.6 ***	43.0 ± 6.3 ***	60.0 ± 8.8 ***	80.3 ± 6.9
9	53.3 ± 4.0 ***	27.5 ± 2.5 ***	58.2 ± 4.3 ***	39.5 ± 5.4 **	28.0 ± 2.5 ***
10	113.1 ± 6.7	57.5 ± 10.8 ***	49.6 ± 5.3 ***	91.4 ± 8.3	102.1 ± 11.8
11	58.9 ± 1.9 ***	12.1 ± 3.2 ***	104.6 ± 6.1	119.1 ± 3.0	93.6 ± 8.3
12	111.9 ± 5.6	34.9 ± 2.3 ***	89.9 ± 5.7	82.8 ± 5.4	79.4 ± 6.9
13	71.4 ± 1.8 ***	63.6 ± 7.9 ***	93.4 ± 3.5	70.7 ± 6.1 **	68.3 ± 6.5 **
14	97.5 ± 1.7	95.1 ± 3.8	162.6 ± 18.1	123.5 ± 5.9	73.1 ± 3.4 *
15	122.6 ± 3.0	83.9 ± 7.1	91.8 ± 13.8	99.3 ± 4.1	108.5 ± 4.7
16	96.5 ± 0.7	94.5 ± 9.4	94.8 ± 11.4	92.1 ± 2.0	96.2 ± 1.8
17	79.5 ± 1.4 **	66.6 ± 5.6 **	140.9 ± 7.9	108.9 ± 3.3	83.4 ± 2.0
18	75.2 ± 2.9***	40.7 ± 7.1 ***	117.4 ± 7.2	131.7 ± 7.7	87.0 ± 3.8

9.2 Compounds **3**, **4** and **5** inhibit iNOS expression and NO production in a dose-dependent manner

All of the compounds that decreased iNOS protein levels by over 90% in the first screening experiments were selected for dose-response studies. Clear dose-response effects were seen with compounds (3), (4) and (5) on iNOS protein expression; the IC50 values lay in a range between 0.3 μM and 3 μM (Figure 36).

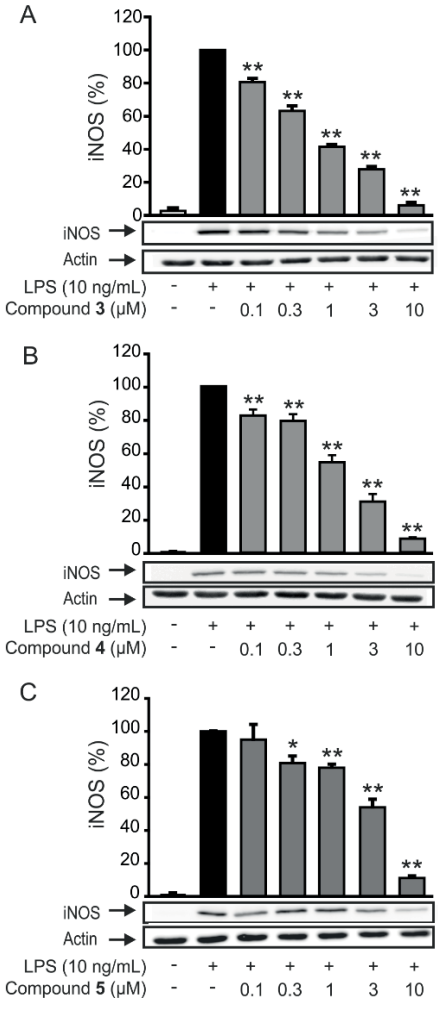


Figure 36. Effects of compounds (**3**), (**4**) and (**5**) on LPS-induced iNOS protein expression in J774 macrophages. iNOS expression was measured by western blot after 24 h incubation with actin as the loading control. Values are expressed as mean + SEM, n=4, * = $p<0.05$ and ** = $p<0.01$ as compared to cells cultured with LPS only. (Reprinted with permission from Laavola et al. 2016 J Nat Prod 79:247-280 Copyright 2016 American Chemical Society)

None of the compounds had any significant effect on iNOS mRNA levels (Figure 37) suggesting that the effect of these betulin derivatives (**3-5**) on iNOS protein expression and subsequently only minor effects on NO production could be mediated through post-transcriptional regulation of iNOS expression.

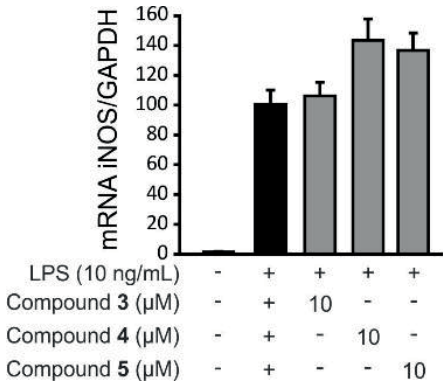


Figure 37. Effects of compounds (**3**), (**4**), and (**5**) on iNOS mRNA levels. J774 macrophages were cultured with LPS alone or with LPS and the compound of interest for 6 h and iNOS mRNA was measured by RT-PCR. The results were normalized against GAPDH mRNA and are expressed as mean + SEM, n=4. (Reprinted with permission from Laavola et al. 2016 J Nat Prod 79:247-280 Copyright 2016 American Chemical Society)

9.3 Pyrazolobetulinic acid (**9**) reduces the expression of the inflammatory genes iNOS, MCP-1 and IL-6 and acute inflammatory response *in vivo*

In the primary screening experiments, compound (**9**) (Figure 38) exerted a statistically significant inhibitory effect on all of the inflammatory factors measured in the first phase (NO, iNOS, COX-2, MCP-1 and IL-6). Therefore, dose-response studies were carried out for the factors that were inhibited by over 50 % by compound (**9**) at a concentration of 10 μM.

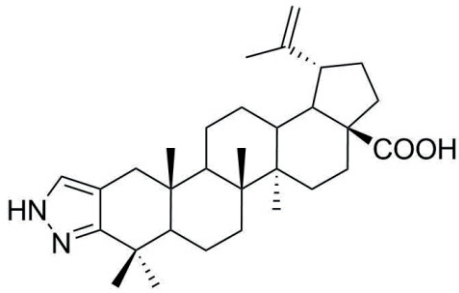


Figure 38. Structure of pyrazobetulinic acid compound (**9**)

Compound (**9**) significantly down-regulated iNOS protein expression and reduced NO, IL-6 and MCP-1 production in J774 cells (Figure 39).

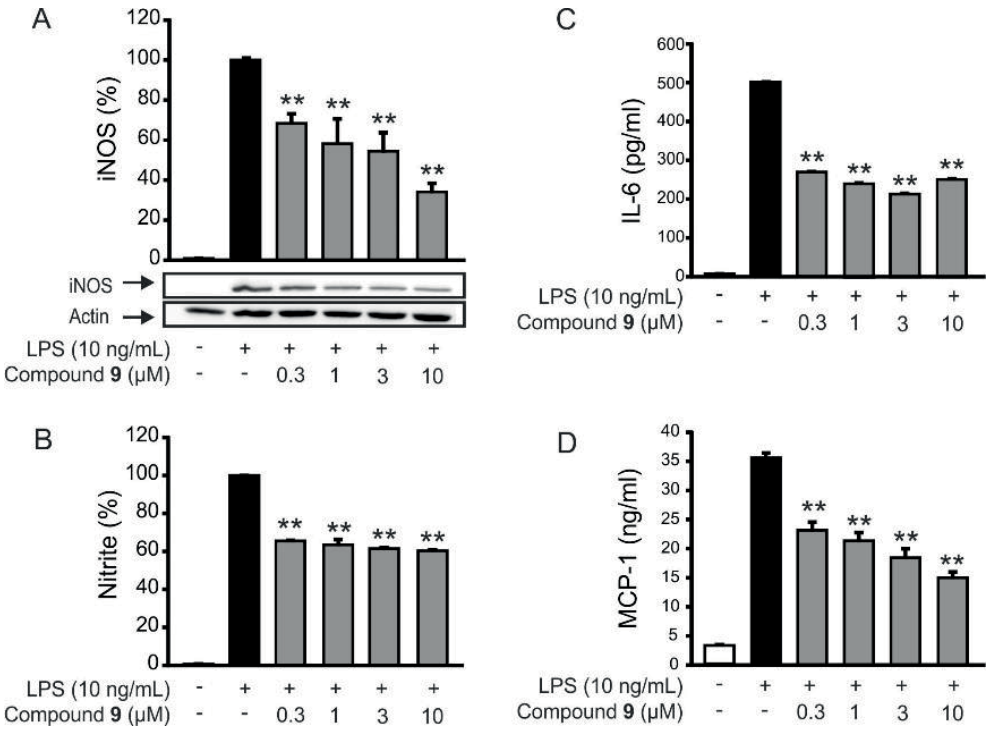


Figure 39. Effects of increasing concentrations of compound (**9**) on LPS-induced iNOS expression (A) and NO (B), IL-6 (C) and MCP-1 (D) production in J774 macrophages. iNOS expression was measured by western blot after 24 h incubation with actin as the loading control, NO production as its stable metabolite nitrite by the Griess reaction and MCP-1 and IL-6 concentrations in the culture media were determined by ELISA. Values are expressed as mean + SEM, n=4, ** = $p<0.01$ as compared to cells cultured with LPS only. (Reprinted with permission from Laavola et al. 2016 J Nat Prod 79:247-280 Copyright 2016 American Chemical Society)

Compound (**9**) suppressed also iNOS mRNA levels significantly at 6 and 24 hours, IL-6 mRNA levels at 6 hours, and MCP-1 mRNA levels at 3 and 6 hours (Figure 40).

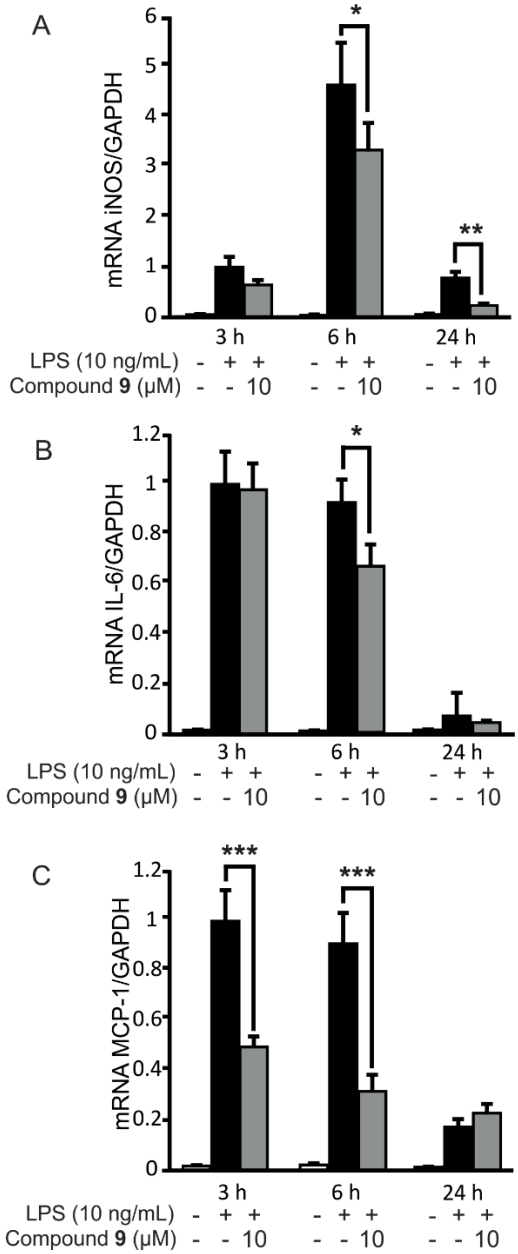


Figure 40. Effects of compound (**9**) on iNOS (A), IL-6 (B) and MCP-1 (C) mRNA levels. J774 macrophages were cultured with LPS alone or with LPS and compound (9) for 3, 6, or 24 h and thereafter total RNA was extracted. mRNA expression was measured by RT-PCR. The results were normalized against GAPDH mRNA and are expressed as mean + SEM, n=4, * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$ as compared to cells cultured with LPS only. (Reprinted with permission from Laavola et al. 2016 J Nat Prod 79:247-280 Copyright 2016 American Chemical Society)

Compound (**9**) was chosen to undergo *in vivo* testing because it had proved to possess important anti-inflammatory effects *in vitro* by modulating several inflammatory genes. As hypothesized, the anti-inflammatory *in vitro* effects were also turned into *in vivo* and compound (**9**) decreased carrageenan-induced paw inflammation in the mouse in a statistically significant manner. Intraperitoneal administration of compound (**9**) at the dose of 10 mg/kg reduced carrageenan-induced paw edema at 3 hours by 27% and at 6 hours by 44% whereas dexamethasone (2 mg/kg) decreased carrageenan-induced paw inflammation by about 65% at 3 and 6 hours (Figure 41).

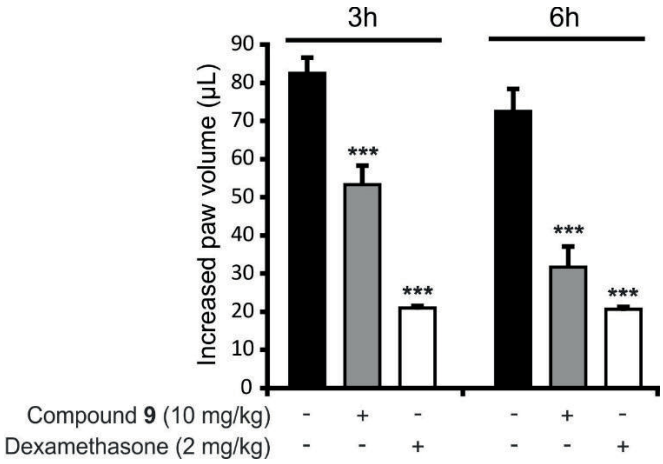


Figure 41. Effect of compound (**9**) on carrageenan-induced paw inflammation in the mouse. Compound (**9**) at the dose of 10 mg/kg or dexamethasone (2 mg/kg) or saline were administered i.p. 2 h prior to carrageenan (1.5%) was injected into the paw. The inflammatory edema was measured 3 and 6 h after the carrageenan injection and was compared to the basal level. Results are expressed as mean + SEM, n=6-12, *** = $p<0.001$ as compared to mice without drug treatment. The contralateral control paw injected with saline developed no measurable edema. (Reprinted with permission from Laavola et al. 2016 J Nat Prod 79:247-280 Copyright 2016 American Chemical Society)

Discussion

10 Methodology

Macrophages are major players in many chronic diseases related to inflammation. Their central and important role in inflammation is widely accepted (Schultze *et al.* 2015). Therefore, most of the *in vitro* tests utilized in the present study were carried out in J774 macrophages. Immortalized cell lines provide a reproducible and straightforward model for experiments investigating complex intracellular mechanisms. J774 mouse macrophages were originally isolated from the ascites of female BALB/c mice with reticulum cell sarcoma (Ralph *et al.* 1975). They are readily adherent cells, which display many of the characteristics of primary macrophages such as lysozyme synthesis and secretion, phagocytosis of carbon particles, expression of Fc receptors, and ingestion of erythrocytes coated with IgG (Muschel *et al.* 1977, Ralph *et al.* 1975). Nonetheless, one needs to be aware that also significant differences may exist in the expression of cell surface proteins and in the cytokine production between cell lines and primary-derived macrophages following exposure to inflammatory stimuli (Chamberlain *et al.* 2009).

Reporter gene assay is a practical and efficient tool for investigating gene regulation. To investigate NF-κB mediated transcription, human embryonic kidney 293 cells and human T/C28a2 chondrocytes were transfected with a reporter construct where luciferase gene expression was controlled by a NF-κB dependent promoter. Macrophages were not used in transfection studies because they are generally difficult to transfect. Macrophages can easily disrupt nucleic acid integrity and make gene transfer inefficient because of their many degradative enzymes (Zhang *et al.* 2009). HEK-293 cells instead are easy to grow in the culture and very straightforward to transfect, which makes them an ideal host for gene expression studies (Lin *et al.* 2015).

T/C28a2 cells were originally established from juvenile costal chondrocytes transfected by SV-40 in 1994 by Goldring et al. (Goldring *et al.* 1994). This immortalized human chondrocyte cell line expresses many cartilage-specific matrix proteins, including type II, type IX, and type XI collagens and aggrecan, and they also exhibit the expected chondrocyte responses to IL-1β, including decreased type II collagen expression and increased expression of matrix metalloproteinases (Goldring *et al.* 1994). Chondrocyte cell lines are easier to transfect with high efficiency than primary chondrocytes. For these reasons, T/C28a2 cells were used in NF-κB mediated transcription studies.

Otherwise, human primary chondrocytes were used because generally it is considered that the phenotype of primary cells is closer to the *in vivo* situation. Chondrocytes retain normal cellular functions and signaling in primary cultures. However, they have also limitations because of their limited life span, restricted number of cells and heterogeneity between donors leading to possible variability in cellular characteristics. There are also practical and ethical reasons limiting the use of primary cells.

New experimental compounds and extracts of natural origin were used in the present study. Therefore, the cytotoxicity of all test compounds was initially investigated with the XTT test in the used cell models. This assay was used to eliminate the possibility that the effects observed could be due to reduced cell viability.

Interesting *in vitro* findings were confirmed in an animal model. The use of animals was limited because of ethical reasons. The carrageenan-induced paw inflammation model in the mouse was chosen because it is a widely used experimental model of acute inflammation in pre-clinical pharmacological studies and in the search for anti-inflammatory drugs (Posadas *et al.* 2004). Carrageenan-induced inflammation is mediated by inflammatory cells, especially macrophages and neutrophils. The development of edema has been described as a biphasic event, where the first phase starts almost immediately after carrageenan injection and lasts about 1-2 hours. During this phase, the reaction is mediated by histamine, serotonin and kinins. Our focus was on the later phase occurring 3-6 h after carrageenan injection. This second phase is associated with local production of prostaglandins and nitric oxide following increases in COX-2 and iNOS expression. During the later phase, levels of various proinflammatory cytokines, such as IL-1β, TNF-α and IL-6 in the inflamed tissue are also elevated (Handy & Moore 1998, Loram *et al.* 2007, Salvemini *et al.* 1996).

Standard cell-biological methods ELISA, multiplex bead array assay, western blotting and quantitative RT-PCR were used to determine protein and mRNA expression, respectively. RT-PCR is a highly specific method used to detect mRNA levels of the gene of interest. GAPDH was used as a housekeeping gene and the levels of the mRNA of interest were normalized against that gene. GAPDH is the most commonly used reference gene; it is known to be constantly expressed in most but not all cellular conditions (Kozera & Rapacz 2013). Western blotting, ELISA and multiplex bead array methods are based on an antigen-antibody reaction which supports the reliability of the results. The amounts of protein loaded on the western blotting gels were controlled by measuring protein concentrations of the samples beforehand and using β-actin as a loading control. multiplex bead array assay is a more efficient method than ELISA because multiple analytes can be measured simultaneously. However, also the risk for cross-reactivity increases when multiple ligands are analyzed at the same time. However, in general terms, the quantitative data obtained from multiplex bead array is comparable to ELISA when variability is minimized by using the same antibodies, diluents etc. (Elshal & McCoy 2006). Standardized protocols and control compound (anti-inflammatory glucocorticoid dexamethasone) were used in the studies.

11 Evaluation of the anti-inflammatory properties of stilbenes, nortrachelogenin and semi-synthetic betulin derivatives

The present study investigated the anti-inflammatory properties of knotwood extract of *Pinus sylvestris*. The extract was found to inhibit LPS induced NO production in activated macrophages in a dose-dependent manner. This was attributable to the extract's inhibitory effect on iNOS expression after inflammatory stimuli as the levels of both iNOS protein and the mRNA were decreased. In addition, a clear reduction was observed in the expression of two inflammatory genes, IL-6 and MCP-1. The immunomodulatory effects of *Pinus sylvestris* knot extract have not been reported earlier. Previously, it has been shown that the extract of *Pinus sylvestris* leaf buds and bark could reduce NO production and iNOS expression in LPS stimulated murine macrophages but exerted no effects on COX-2 expression while the bark extract also reduced PGE_2 production (Karonen *et al.* 2004, Vigo *et al.* 2005). However, in these previous studies, the active constituents were not identified. Previously, it had been reported that the same pine knot extract and its compounds had antiproliferative and proapoptotic effects in prostate cancer cells and also a three week daily ingestion of the extract displayed anti-tumorigenic efficacy *in vivo* in orthotopic PC-3M-luc2 xenografts in athymic mice (Yatkin *et al.* 2014).

In further studies, the stilbene components of pine knot extract, i.e. pinosylvin (5 % w/w) and monomethylpinosylvin (12 % w/w), were shown to have qualitatively similar anti-inflammatory effects as the pine knot extract. This suggested that it is likely that they contribute to the anti-inflammatory effects of the extract. Because resveratrol had been previously proved to have immunomodulatory effects (Poulsen *et al.* 2015), it was added to the tests as a control stilbenoid compound. Furthermore, two other stilbenes astringin and isorhapontin were investigated because they are present in Norway spruce, another Nordic conifer. Our *in vitro* results confirmed the previous finding about resveratrol and also revealed that resveratrol reduced inflammatory paw edema in the mouse. This kind of effect was not detected in the previous study of Gentilli et al., but the difference in the dosing time [shortly before (by Gentilli et al.) vs 2 h before (as in the present study) carrageenan] of resveratrol

may explain these discrepant results. Furthermore, the present results show that the anti-inflammatory effects of resveratrol are shared by pinosylvin and monomethylpinosylvin and interestingly, pinosylvin and monomethylpinosylvin were more potent than resveratrol in combatting carrageenan-induced paw inflammation. The present pinosylvin *in vitro* results were supported by the previous reports in different models (Lee *et al.* 2006, Park *et al.* 2004, Park *et al.* 2011). By the time our results were published, no previous data on the anti-inflammatory effects of monomethylpinosylvin had been reported.

Pinosylvin is structurally close to resveratrol. Resveratrol has only one additional hydroxyl group compared to pinosylvin. Monomethylpinosylvin instead differs with one methyl group from pinosylvin. The effect of these three stilbenoids were qualitatively similar and no major differences based on their structure could be detected. There are only a few published studies on the structure-activity relationship of stilbenes. Those have concluded that the double bond between the two rings is not important and the activity is inhibited when oxygen groups are replaced with a hydrogen group whereas a balance of oxygen groups between the benzene rings is required for the biological activity (Cho *et al.* 2002, Kageura *et al.* 2001, Matsuda *et al.* 2000). In the previous study, the presence of a glucoside moiety was also found to reduce anti-inflammatory activity which explains also our finding that astringin and isorhapontin, both glycosylated stilbenoids, had no immunomodulatory effects in activated macrophages perhaps due to their poor transportation into the cell (Matsuda *et al.* 2000).

In a very recent publication from our research group, five semi-synthetic pinosylvin derivatives were investigated. The presence of a small substitute in the R1 position in the left-hand side phenyl ring did not exert any significant effect on anti-inflammatory potential whereas longer substituents seemed to attenuate the effect with the overall conclusion being that semi-synthetic derivatives were less potent than natural pinosylvin. (Erasalo *et al.* 2018)

Z-and E-resveratrol are known metabolic products of pinosylvin (Roupe *et al.* 2005). Therefore it is possible that pinosylvin may induce some of its anti-inflammatory effects via metabolism to resveratrol.

Lignans are biogenetically closely related to stilbenoids and are synthesized through the same phenylpropanoid pathway in plants (Harmatha *et al.* 2011). In chemical terms, the lignan group is heterogenic and diverse from stilbenes. Nortrachelogenin is a classical lignin, a member of the dibenzylbutyrolactone lignans and also a major component (7 % w/w) of the pine knot extract. The effect of nortrachelogenin on iNOS protein expression and MCP-1 production was more

powerful whereas on NO production, its effects were clearly less than that of stilbenoids. The anti-inflammatory properties of nortrachelogenin can also partly contribute to the effects of pine knot extract in addition to stilbenoids. As far as we are aware, the present study was the first to apply a systematic approach to evaluate the anti-inflammatory potential of nortrachelogenin. Our findings are supported by a previous study where six different lignans, secoisolariciresinoldiglucoside, secoisolariciresinol, pinoresinol, lariciresinol, matairesinol and hydroxymatairesinol, were investigated. Pinoresinol was found to be the most effective in reducing IL-6, MCP-1 and PGE_2 production in IL-1β stimulated Caco-2 cells (During *et al.* 2012). Structurally pinoresinol belongs to the tetrahydrofuro-furan lignans and it is known to be converted to enterolactone in the colon whereas nortrachelogenin is not metabolized to enterolactone (Saarinen *et al.* 2002).

Interestingly, the complex pentacyclic structure of betulin derivatives is close to the tetracyclic structure of the very well-known and powerful anti-inflammatory drugs, glucocorticoids. Two-types of anti-inflammatory compounds were found from the tested semi-synthetic betulin derivatives. The novel findings were that compounds (**3**), (**4**) and (**5**) showed a selective effect on iNOS expression and that pyrazolobetulinic acid (**9**) reduced acute inflammation *in vivo* and downregulated the expression of inflammatory genes iNOS, COX-2, IL-6 and MCP-1. Betulin (**1**) has previously been reported to inhibit IL-6 production in RAW 264.7 macrophages which was different to our finding in J774 macrophages (Wu *et al.* 2014). The difference could be related to the higher concentration used or the different responses of different immortalized macrophage cell lines. The previously reported findings that betulinic acid exerted effects on NO and IL-6 production in RAW 264.7 and peritoneal macrophages (Costa *et al.* 2014, Ju *et al.* 2015) were confirmed in the present study. In addition, we extended the previous data by showing the anti-inflammatory effects of 16 newly synthesized betulin derivatives.

The conclusion of structure activity relationship of the semi-synthetic betulin derivatives on iNOS inhibitory activity were (III):

- modifying the hydroxymethyl group of betulin structure at position C-28 by converting it to an oxime moiety (**3**) enhanced the downregulation of iNOS
- fusing a heterocyclic group to the A ring of betulonic acid (**6**) increased the inhibitory activity
- the additions of a fused pyridine (**11**) or pyrazine (**4**) ring to the A ring of the betulinic acid (**2**) skeleton increased the inhibitory activity

- changing the carboxyl group at position C-28 to a primary amide group of the pyrazine derivative (**12**) resulted in suppressed activity
- the isoxazole derivative of dihydrobetulonic acid (**7**) displayed excellent activity, which was further improved by changing its carboxyl group to a primary amide group as in compound (**5**) and isopropyl to isopropenyl moiety at position C-20
- when the primary amide group of the compound (**5**) was changed to a formyl group (**10**), activity decreased

Based on our results, no clear correlation between the derivatives' structure and the suppressive effect on COX-2 expression could be found because only a few compounds exhibited any inhibitory activity on COX-2 expression.

12 Comparison of the pharmacological mechanisms of stilbenes, nortrachelogenin and semi-synthetic betulin derivatives in the inhibition of inflammatory genes

All compounds investigated in detail in this study [pine knot extract, pinosylvin, monomethylpinosylvin, nortrachelogenin and betulin derivatives (**3-5**) and (**9**)] inhibited iNOS protein expression. The present study demonstrated that pine knot extract, pinosylvin, monomethylpinosylvin and pyrazolobetulinic acid (**9**) suppressed similarly iNOS mRNA, which evidence that a transcriptional mechanism is likely to explain this inhibitory effect on iNOS-NO pathway. NF-κB is a significant transcription factor regulating iNOS transcription (Pautz *et al.* 2010). We found that NF-κB mediated transcription was inhibited by the extract of *Pinus sylvestris* and its stilbene constituents: pinosylvin and monomethylpinosylvin, which may, at least partly, explain their inhibitory effects on the expression of iNOS and other NF-κB dependent genes.

According to previous studies, HO-1 plays an important role in inflammation by regulating the functions of antigen-presenting cells, dendritic cells, and regulatory T cells. HO-1 is anti-inflammatory and inhibitits NF-κB (Juan *et al.* 2005, Ryter & Choi 2015). During our investigation, we found that the pine knot extract and its active stilbenes increased HO-1 expression in activated macrophages. This may represent a putative mechanism for their anti-inflammatory action and explain how they were able to decrease the NF-κB activity and reduce the expressions of inflammatory genes. Interestingly, Bauerova et al. recently reported results supporting our study, where pinosylvin increased HO-1 expression in the lung tissue and inhibited NF-κB activation in lung and liver tissue in an adjuvant arthritis model in the rat (Bauerova *et al.* 2015). There is also previous data of some other natural compounds increasing HO-1 (Motterlini & Foresti 2013). Furthermore, one previous study of resveratrol supports our original finding on the protective effects of pinosylvin and monomethylpinosylvin being mediated through HO-1 expression. Juan and coworkers showed that resveratrol enhanced HO-1 expression in human aortic smooth muscle cells (Juan *et al.* 2005).

Another recent finding is that pinosylvin down-regulates Akt phosphorylation, a marker for PI3K activity, in J774 macrophages (Erasalo *et al.* 2018). This opens a new hypothesis for the link between the Akt and NF-κB. Another interesting published finding is that pinosylvin increases glucose uptake by up-regulating SIRT1 activity by stimulating 5`-adenosine monophosphate activated protein kinase (AMPK) since AMPK is dysregulated in inflammation (Jeon 2016, Modi *et al.* 2017).

Pyrazolobetulinic acid (**9**) repressed also IL-6 and MCP-1 mRNA levels in addition to iNOS mRNA. All these genes have also been shown to be regulated by NF-κB (Pautz *et al.* 2010, Schaper & Rose-John 2015, Ueda *et al.* 1997). Further investigations will be needed to clarify the detailed mechanisms of the pyrazolobetulinic acid (**9**).

Nortrachelogenin and betulin compounds (**3**), (**4**) and (**5**) showed suppressed iNOS protein levels but had less effect on NO production and did not alter iNOS mRNA levels suggesting a post-transcriptional mechanism on iNOS expression. Most known iNOS inhibitors regulate iNOS expression at the transcriptional level but there are also previous data that some PPARα agonists, natural compound curcumin, and lignan compound arctigenin promote degradation of iNOS through the proteasome pathway (Ben *et al.* 2011, Paukkeri *et al.* 2007, Yao *et al.* 2012). In the present study, the proteasome inhibitor lactacystin reversed the effect of nortrachelogenin on iNOS protein expression, strongly suggesting that nortrachelogenin enhances iNOS protein degradation through the proteasome pathway leading to reduced iNOS levels and suppressed NO production. Further studies will be needed to clarify in more detail which proteasome subcomponents are targets of nortrachelogenin. At least 26S and 20S have been reported to be important for the degradation of iNOS and both are selectively inhibited by lactacystin (Fenteany & Schreiber 1998, Jin *et al.* 2009, Musial & Eissa 2001).

More detailed mechanistic studies on betulin derivatives were beyond the scope of this thesis but possible post-transcriptional targets could be a specific class of noncoding RNAs, microRNAs. There are a few identified miRNAs that regulate human and mouse iNOS gene expression. miRNA-939 has been reported to regulate iNOS expression in human hepatocytes by decreasing cytokine induced iNOS protein expression while having no effect on iNOS mRNA levels or mRNA stability (Guo *et al.* 2012). miR-146a has been reported to down-regulate both human and mouse iNOS genes and miR-26a also the human iNOS gene (Guo & Geller 2014). As far as we are aware, no anti-inflammatory drugs have been shown to inhibit iNOS expression through this kind of mechanism but it is possible that small molecules

directly modulate miRNAs, leading to reduced translation and / or mRNA degradation (Jeker & Marone 2015).

In addition to iNOS, nortrachelogenin exerted a dose-dependent inhibitory effect on PGE_2 production but had no effect on COX-2 expression. Interestingly, nortrachelogenin reduced the expression of mPGES-1 adding it to the few compounds known to down-regulate this inducible enzyme responsible for significantly enhanced PGE_2 production in inflammation (Korotkova & Jakobsson 2014). This is a novel finding linked to nortrachelogenin and an interesting mechanism to selectively suppress the synthesis of PGE2, the most important prostanoid in inflammation, while leaving the physiologically important production of other prostanoids intact. In addition, nortrachelogenin may inhibit the activity of the PGE_2 synthesizing enzymes or the release of arachidonic acid, which represents the rate-limiting step in eicosanoid biosynthesis. Furthermore, nortrachelogenin reduced also IL-6 production in LPS activated macrophages. Two-way interactions between PGE_2 and IL-6 have been described in different cell lines and this kind of effect may have occurred also in our experiments (Dendorfer *et al.* 1994, Liu *et al.* 2006, Wang *et al.* 2011).

Interestingly, our *in vitro* findings were confirmed as we detected an anti-inflammatory effect also *in vivo*. The carrageenan-induced inflammatory response in the paw has been reported to be mediated partly by increased NO production, since it has been shown to be sensitive to the anti-inflammatory properties of iNOS inhibitors (Handy & Moore 1998, Salvemini *et al.* 1996). In our study, the inhibitory action of pinosylvin and monomethylpinosylvin was comparable to the iNOS inhibitor L-NIL as well as to dexamethasone. Our results suggest that pinosylvin and monomethylpinosylvin reduced the acute inflammatory response *in vivo,* possibly via a mechanism involving the stimulation of HO-1 levels leading to inhibition of NF-κB activity and ultimately to the suppression of inflammatory gene expression including iNOS. In addition, it has been shown recently that pinosylvin inhibits IL-6 and MCP-1 production in the paw inflammation model (Erasalo *et al.* 2018). Additionally, it has been reported by Moilanen et al. that TRPA1 also mediates carrageenan-induced inflammation. Carrageenan-induced response was shown to be attenuated in TRPA1 deficient mice (Moilanen *et al.* 2012). Pinosylvin (Moilanen *et al.* 2016) and resveratrol have been reported to be potent inhibitors of TRPA1 *in vitro* and *in vivo* whereas monomethylpinosylvin did not have a similar effect (Yu *et al.* 2013). Therefore resveratrol's effect on carrageenan paw inflammation could also be mediated partly through TRPA1 inhibitor activity, which may be an independent

mechanism or associated with the mechanisms and factors identified in the present study.

A neutralizing anti-PGE_2 antibody as well as nonsteroidal anti-inflammatory drugs have been shown to significantly diminish carrageenan-induced paw edema (Portanova *et al.* 1996), supporting the concept that reduced PGE_2 formation, in addition to inhibition of the iNOS-NO pathway, by nortrachelogenin contributes to its anti-inflammatory action *in vivo*. Nortrachelogenin and other studied compounds also inhibited MCP-1 production which could lead to reduced numbers of inflammatory cells to be recruited to the inflammation site.

Pyrazolobetulonic acid (**9**) suppressed the expression of several inflammatory factors, i.e. iNOS, IL-6 and MCP-1 at both the protein and mRNA levels, suggesting that it caused interference with a transcriptional mechanism. The effect could be mediated via NF-κB or other relevant transcription factors e.g. AP-1, IRF-1, SP-1 or STAT-1 which are all involved in the regulation of the genes iNOS, IL-6 and MCP-1 (Pautz *et al.* 2010, Tanaka *et al.* 2014, Ueda *et al.* 1994). The anti-inflammatory properties of compound (**9**) in carrageenan-induced inflammation were possibly mediated by inhibition of iNOS and COX-2 expression and suppression of the production of NO, IL-6, MCP-1 and prostanoids.

13 Importance of IL-6 in OA and chondroprotective properties of the pine knot extract and its components

IL-6 levels in synovial fluid samples of the OA patients were significantly higher than those in plasma and correlated with the amounts of MMP enzymes and disease severity. The plasma concentrations were at the same levels as those reported in healthy individuals (Hunter & Jones 2015). There is one previous report that (n=78) synovial fluid levels of IL-6 were significantly higher in patients with a cartilage defect or OA than in donors without joint pathology (Tsuchida *et al.* 2014). In another study (n=34), a similar finding was made in IL-6 synovial fluid levels between donors without joint pathology and OA patients (Beekhuizen *et al.* 2013).

In a follow-up study published in 2009, it was proposed that serum concentrations of IL-6 in OA patients are related to joint damage observed in radiographs. In a study examining 908 healthy women, radiographic knee OA status was assessed by Kellgren-Lawrence grade at baseline and subsequently after 10 and 15 years. Serum IL-6 levels were clearly associated with the development of radiographic knee OA (Livshits *et al.* 2009).

Fernandes et al. studied IL-6 polymorphism (rs1800796) in elderly hip and knee OA patients (n=257). The presence of the C allele predicted lower susceptibility of OA compared to G allele. Patients with genotype GC and CC had significantly lower serum IL-6 levels compared to patients with the GG genotype (Fernandes *et al.* 2015). All these previous clinical studies together with the present findings underline the important role of IL-6 in OA.

In the present study, pine knot extract and pinosylvin, monomethylpinosylvin, and resveratrol were found to inhibit IL-6 production and increase aggrecan mRNA expression in human primary chondrocytes stimulated with the OA related cytokines IL-1β or IL-17. These kinds of effects would be beneficial in maintaining cartilage homeostasis. The increased aggrecan expression might also be a positive consequence of IL-6 inhibition because IL-6 has been shown to inhibit cartilage matrix formation, especially proteoglycan production, at least in murine bone marrow-derived mesenchymal stem cells (Wei *et al.* 2013).

Pinosylvin has been shown to be effective in an adjuvant arthritis model (Drafi *et al.* 2012, Jancinova *et al.* 2012, Macickova *et al.* 2010), which has been so far the only

inflammatory model used *in vivo* to clarify the effects of pinosylvin. Otherwise, there is a very limited amount of data available on the effects of stilbenes on OA. There is one study where intra-articular injections of resveratrol prevented cartilage degradation in a surgical mouse model of OA and the effects of resveratrol were also studied in human primary chondrocytes. Resveratrol inhibited iNOS and MMP-13 mRNA expression and increased collagen II and similar to our study, also the expression of aggrecan mRNA. Resveratrol significantly induced the activation of SIRT1 and reduced HIF-2α levels in mouse OA cartilage and in IL-1β-treated human chondrocytes (Li *et al.* 2015). In another study, resveratrol reduced MMP-3, ADAMTS-4 and IL-6 mRNA levels, again supporting our finding (Schwager *et al.* 2015). We showed for the first time that also pine knot extract, pinosylvin and monomethylpinosylvin could inhibit IL-6 production and increase aggrecan expression in primary human OA chondrocytes.

Interestingly, it has been recently reported that monomethylpinosylvin inhibits pain behavior induced by capsaicin, a known TRPV1 activator, and that pinosylvin inhibits TRPA1-induced calcium influx *in vitro* and TRPA1 mediated paw inflammation in mice (Moilanen *et al.* 2016, Yu *et al.* 2013). Therefore, based on the present and published findings, it is tempting to speculate that pine knot stilbenoids could possibly be disease-modifying OA drug candidates, which might have also pain-relieving properties.

14 Wood biochemicals as possible drug candidates in inflammatory diseases

The discovery and development of a new pharmaceutical product is an extremely expensive and long-lasting project. Despite massive recent efforts and improved screening methodologies, the number of new drugs has not increased. The trend has been to develop single-targeted and highly specific agents (Lu *et al.* 2012). Because organisms easily develop compensatory mechanisms, it is not always possible to achieve the desirable effect by only aiming at one specific target. That is why combinatorial therapy is needed in many complex diseases. This leads to the suggestion that there may be benefits to adopting a multi-target approach for drug design. Many natural products are multi-target agents (Lu *et al.* 2012). So far, only a small part of the higher plants have been pharmacologically investigated (Cragg & Newman 2013) and it is reasonable to assume that there is a treasure-trove of untapped potential to be discovered from nature.

Natural products have many beneficial properties; they have often lower hydrophobicity and greater stereochemical content compared to synthetic compounds (Stratton *et al.* 2015). On the other hand, their low bioavailability has led to poor success in clinical trials (Watkins *et al.* 2015). Naturally occurring compounds can be absorbed inadequately in the gastrointestinal tract or alternatively, they might be rapidly metabolized and excreted (Mobasheri 2012). Many of these bioavailability problems can be improved by using drug delivery systems based on nanotechnology (Bonifacio *et al.* 2014). As found out in the present study with betulin derivatives, the compounds can be chemically modified to produce more suitable candidates for pharmaceutical applications.

In summary, the results obtained in this study suggest that wood-derived compounds could be used for drug discovery of new anti-inflammatory agents. The usage of the entire extract may be more effective in some cases but may increase the risk of adverse events, which are major problems in medicine (Lu *et al.* 2012). Lignans and stilbenoids can be extracted from pine knots in economically reasonable amounts as can betulin from birch bark. All of the compounds studied here are currently waste products of mechanical forest industry and paper and paper and pulp industry without any meaningful use. This could be one way of increasing the use

and value of wood material according to one strategic goal of Finnish bioeconomic policy (Ollikainen 2014). A summary of the detected immunomodulatory effects of the studied wood biochemicals is presented in Figure 42.

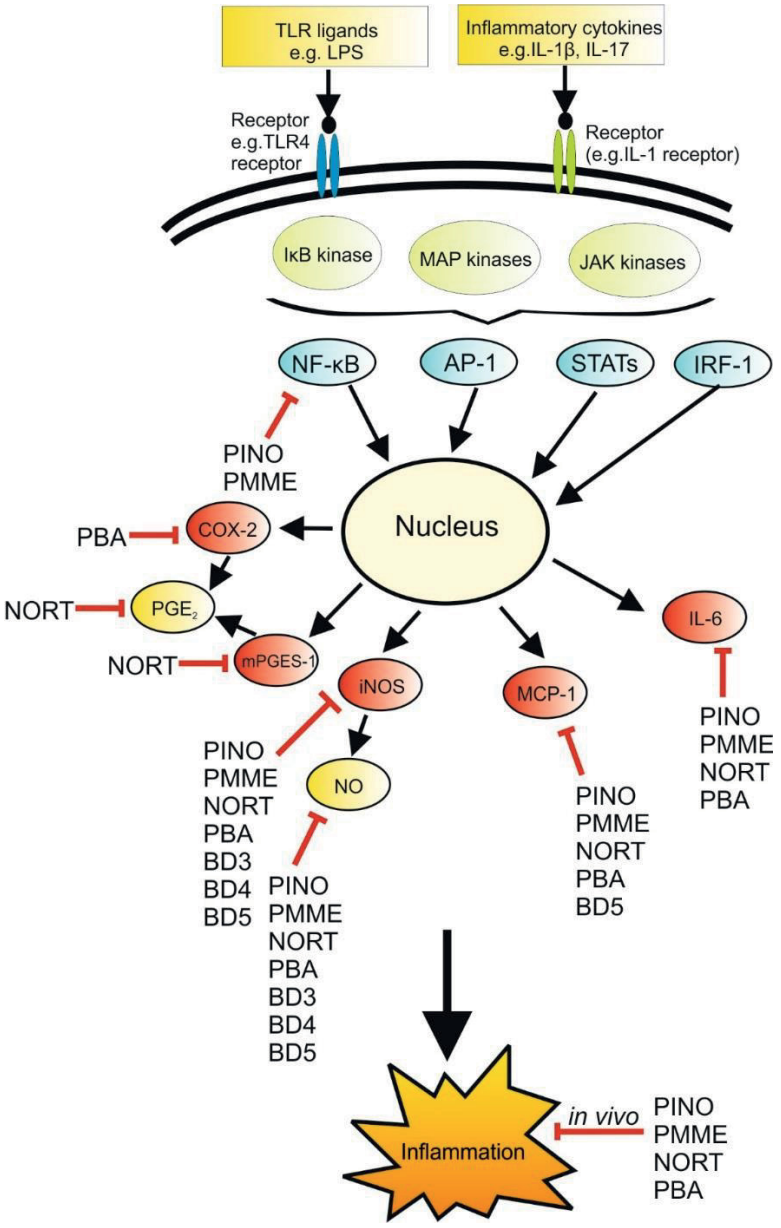


Figure 42. Summary of the immunomodulatory effects of the wood biochemicals found in the present study (PINO=pinosylvin, PMME=monomethylpinosylvin, NORT=nortrachelogenin, PBA=pyrazolobetulinic acid (betulin derivative 9), BD3-BD5=betulin derivatives 3-5)

Summary and conclusions

The objectives of the present study were to identify anti-inflammatory compounds in the pine knot and to screen immunomodulatory properties of semi-synthetic betulin derivatives.

The major findings are as follows:

1. Pine knot extract, and its major stilbenoid constituents pinosylvin and monomethyl pinosylvin had anti-inflammatory properties both *in vitro* and *in vivo* possibly via a mechanism involving the up-regulation of HO-1 levels leading to inhibition of NF-κB activity and finally to the suppression of inflammatory gene expression.

2. Nortrachelogenin was found to be a promising novel anti-inflammatory compound, downregulating iNOS/NO pathway in macrophages through enhancing iNOS protein degradation in proteasome and by attenuating acute inflammatory response in vivo. Interestingly, nortrachelogenin also suppressed mPGES-1/PGE_2 pathway adding nortrachelogenin to the few known compounds able to downregulate this inducible enzyme responsible for significantly enhanced production of the proinflammatory and hyperalgesia-inducing prostanoids PGE_2 in inflammation.

3A. In a preliminary screening of semi-synthetic betulin derivatives, three novel compounds 3, 4 and 5 were identified; they inhibited iNOS expression and NO production. In further studies, the compounds decreased iNOS expression in a post-transcriptional and dose-dependent manner.

3B. A novel anti-inflammatory betulin derivative, pyrazolobetulinic acid (9), was found to suppress the expression of genes coding for inflammatory enzymes iNOS and COX-2 as well as the production of pro-inflammatory factors

NO, IL-6 and MCP-1 in macrophages *in vitro* and to exert an evident anti-inflammatory effect also *in vivo*.

4. In OA patients, IL-6 levels in synovial fluid were higher than those in plasma; they correlated with the levels of MMP enzymes and radiologically determined disease severity. Interestingly, pine knot extract, pinosylvin and monomethylpinosylvin suppressed IL-6 production and increased aggrecan expression in primary human chondrocytes stimulated with the OA driving cytokines IL-1β and IL-17, shifting the balance between inflammatory and anabolic mechanisms in a beneficial direction.

The present study examined novel wood-derived anti-inflammatory compounds nortrachelogenin and semi-synthetic betulin derivatives and obtained new information on the immunomodulating effects of wood stilbenoids pinosylvin and monomethyl pinosylvin. In addition, it revealed the potential of pinosylvin and monomethyl pinosylvin as disease-modifying compounds in OA chondrocytes and demonstrated the fact that wood biochemicals represent valid starting compounds for drug discovery.

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Original communications

PUBLICATION I

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Pinosylvin and Monomethylpinosylvin, Constituents of an Extract from the Knot of *Pinus sylvestris*, Reduce Inflammatory Gene Expression and Inflammatory Responses in Vivo

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ABSTRACT: Scots pine (*Pinus sylvestris*) is known to be rich in phenolic compounds, which may have anti-inflammatory properties. The present study investigated the anti-inflammatory effects of a knot extract from *P. sylvestris* and two stilbenes, pinosylvin and monomethylpinosylvin, isolated from the extract. Inflammation is characterized by increased release of pro-inflammatory and regulatory mediators including nitric oxide (NO) produced by the inducible nitric oxide synthase (iNOS) pathway. The knot extract (EC_{50} values of 3 and 3 μg/mL) as well as two of its constituents, pinosylvin (EC_{50} values of 13 and 15 μM) and monomethylpinosylvin (EC_{50} values of 8 and 12 μM), reduced NO production and iNOS expression in activated macrophages. They also inhibited the production of inflammatory cytokines IL-6 and MCP-1. More importantly, pinosylvin and monomethylpinosylvin exerted a clear anti-inflammatory effect (80% inhibition at the dose of 100 mg/kg) in the standard in vivo model, carrageenan-induced paw inflammation in the mouse, with the effect being comparable to that of a known iNOS inhibitor L-NIL. The results reveal that the Scots pine stilbenes pinosylvin and monomethylpinosylvin are potential anti-inflammatory compounds.

KEYWORDS: *Pinus sylvestris, stilbene, anti-inflammatory, iNOS, HO-1*

INTRODUCTION

Trees are rich in polyphenolic compounds and could well represent an abundant source of immunomodulatory compounds. Extracts of Scots pine (*Pinus sylvestris*) have been used in traditional medicine as anti-inflammatory treatments for rheumatoid arthritis and in some other inflammatory conditions. However, little is known about the most potent constituents of the extracts or about the precise effects or mechanisms of action in inflammation. Knots, that is, the part of the branches embedded in the stem, also called branch roots, are known to be a rich source of several polyphenols, such as lignans, flavonoids, and stilbenes;[1] for example, the knots in Scots pine (*P. sylvestris*) contain at least two stilbenes, pinosylvin and monomethylpinosylvin (Figure 1).[2] Pinosylvin has been reported to possess antibacterial and antifungal properties that protect the tree from microbial attack and promote the wound-healing process.[3] There are only a few reports describing the therapeutic effects of pinosylvin or monomethylpinosylvin, although another structurally closely related stilbenoid, resveratrol, has attracted much more research interest in recent years.[4−6] Resveratrol is particularly abundant in grapes and peanuts and has been shown to have anti-inflammatory, cancer preventive, and cardioprotective properties.[7] Therefore, we hypothesized that the knot extract of *P. sylvestris* and in particular two of its stilbene constituents, pinosylvin and monomethylpinosylvin, might possess beneficial anti-inflammatory properties.

Inflammation is a host defense mechanism that protects the body against pathogens and irritants and is also involved in removing and healing the consequences of tissue injury. The inflammatory response is usually beneficial, but, should it be inappropriately directed, dysregulated, or prolonged, it may cause injury or disease. Inflammation triggers the induction of an array of inflammatory genes, cytokines, enzymes, and other factors not only in macrophages but also in other inflammatory and tissue cells. Inducible nitric oxide synthase (iNOS) is an important example of an inflammatory gene; that is, it is the enzyme that produces nitric oxide (NO). NO is a signaling molecule that is synthesized from the amino acid L-arginine in a reaction catalyzed by nitric oxide synthase (NOS).[8] Three isoforms of NOS have been characterized; two constitutive NOS isoforms were first detected in endothelial cells (eNOS) and in neurons (nNOS),[9] and there is an inducible isoform, iNOS, which was originally discovered in murine macrophages.[10] NO is involved in the regulation of many physiological and pathophysiological processes including vascular tone, platelet aggregation, immune response, and neurotransmission.[11] In inflammation, NO and NO-derived radicals represent one of the endogenous antimicrobial defense systems.[11] On the other hand, increased iNOS expression and NO production in macrophages and other cells are involved in the pathogenesis of many chronic inflammatory diseases such as asthma and arthritis.[12]

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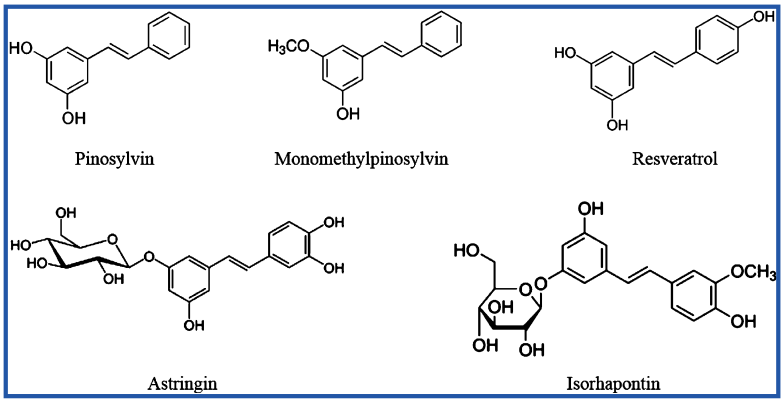

Figure 1. Chemical structures of pinosylvin, monomethylpinosylvin, resveratrol, astringin, and isorhapontin.

Table 1. Primer and Probe Sequences

gene	oligonucleotide	sequence 5′→3′
murine iNOS	forward primer	CCTGGTACGGGCATTGCT
	reverse primer	GCTCATGCGGCCTCCTT
	probe	CAGCAGCGGCTCCATGACTCCC
murine HO-1	forward primer	CCCTCACAGATGGCGTCACT
	reverse primer	GCGGTGTCTGGGATGAGCTA
	probe	CCTGCAGAGACACCCCGAGGGA
murine GAPDH	forward primer	GCATGGCCTTCCGTGTTC
	reverse primer	GATGTCATCATACTTGGCAGGTTT
	probe	TCGTGGATCTGACGTGCCGCC

The aim of the present study was to investigate the anti-inflammatory properties of the knot extract of *P. sylvestris* and to identify the active constituents and molecular mechanisms behind the immunomodulatory effects. The results reveal that two pine stilbenes, pinosylvin and monomethylpinosylvin, are powerful anti-inflammatory compounds both in vitro and in vivo.

MATERIALS AND METHODS

Chemicals. A knotwood extract of *P. sylvestris* and the purified stilbenes, pinosylvin and monomethylpinosylvin, were prepared at Åbo Akademi University. Knots removed from pine stems were freeze-dried and ground to pass through a 2 mm screen. The ground dry knot material was extracted in an accelerated solvent extractor (ASE) apparatus in two stages: (1) with hexane at 90 °C for 3 × 5 min to remove most of the lipophilic extractives and (2) with ethanol/water (95:5 by vol) at 100 °C for 3 × 5 min. The second extract containing primarily the hydrophilic extractives was evaporated to dryness in a vacuum. This extract was used in the biotests. The extract was analyzed by gas chromatography (GC) and gas chromatography−mass spectrometry (GC-MS) after silylation (1). The extract contained the following identified components, in wt % of dry knot material: monomethylpinosylvin, 12%; resin acids, 12%; oxidized resin acids, 11%; pinosylvin, 5%; nortrachelogenin (NTG), 7%; and matairesinol, 2%.

Pinosylvin and methylpinosylvin were isolated from the hydrophilic knot extract using flash chromatography (Biotage Flash 40i, Biotage AB, Uppsala, Sweden) with silica columns, using a step gradient with cyclohexane/acetone. Further purification was achieved by crystallization. The purity of the pinosylvin determined by GC was 89% and that of methylpinosylvin, 94%.

Resveratrol was bought from Tocris Bioscience (Ellisville, MO, USA), and astringin and isorhapontin were from Polyphenols Laboratoires AS (Sandnes, Norway). Rabbit polyclonal iNOS (sc-650), COX-2 (sc-1745), β-actin (sc-1616-R), and HO-1 (sc-1797) antibodies and HPR-conjugated goat polyclonal anti-rabbit antibody and donkey polyclonal anti-goat antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). All other reagents were from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

Cell Culture. Murine J774 macrophages (American Type Culture Collection, Rockville, MD, USA) were cultured at 37 °C in 5% CO_2 atmosphere and grown in Dulbecco's modified Eagle's medium (DMEM) with glutamax-I containing 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/mL), and amphotericin B (250 ng/mL) (Invitrogen, Paisley, UK). Cells were seeded on 96-well plates for the XTT-test and on 24-well plates for the measurements of NO, MCP-1, and IL-6 production and HO-1 and iNOS expression. Cell monolayers were grown for 72 h to confluence before the experiments were started, and the compounds of interest were added in fresh culture medium. Any potential cytotoxicity of the tested compounds was determined by measuring cell viability using the Cell Proliferation Kit II (Roche Diagnostics, Mannheim, Germany).

Preparation of the Stable HEK293pGL4.32NFκB Cell Line. To investigate drug effects on the activation of transcription factor NF-κB and on NF-κB-mediated transcription, the HEK-293 cells (ATCC, Manassas, VA, USA) were stably transfected with a luciferase reporter construct, pGL4.32[*luc2P*/NF-κB-RE/Hygro]. The plasmid was purchased from Promega Corp. (Madison, WI, USA) and contained five copies of an NF-κB response element that drives transcription of the luciferase reporter gene.

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Nitrite Assays. NO production was determined by measuring the accumulation of nitrite, a stable metabolite of NO in aqueous milieu, by the Griess reaction.[13]

Western Blot Analysis. At the indicated time points, cells were rapidly washed with ice-cold phosphate-buffered saline (PBS) and solubilized in cold lysis buffer containing 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 1% Triton-X-100, 0.5 mM phenylmethanesulfonyl fluoride, 1 mM sodium orthovanadate, 20 μg/mL leupeptin, 50 μg/mL aprotinin, 5 mM sodium fluoride, 2 mM sodium pyrophosphate, and 10 μM *n*-octyl-β-D-glucopyranoside. After incubation for 15 min on ice, the lysates were centrifuged (12000*g*, 4 °C for 10 min), and the supernatants were collected and stored in SDS sample buffer at −20 °C. An aliquot of the supernatant was used to determine the protein concentration according to the Coomassie blue method.[14]

Protein samples (20 μg of lysates) were analyzed according to the standard Western blotting protocol as described previously.[15] The membrane was incubated with the primary antibody in the blocking solution overnight at 4 °C and with the secondary antibody in the blocking solution for 1 h at room temperature. Bound antibody was detected using SuperSignal West Pico or Dura chemiluminescent substrate (Pierce, Rockford, IL, USA) and Image Quant LAS 4000 mini imaging system (GE Healthcare Bio-Sciences AB, Uppsala Sweden). The quantitation of the chemiluminescent signal was carried out with Imaging Quant TL software (GE Healthcare).

RNA Extraction and Quantitative RT-PCR. Total RNA was extracted with the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). Reverse transcription of RNA to cDNA and PCR reactions were carried out as previously described.[16] Primers and probes (Table 1) for iNOS, heme oxygenase-1 (HO-1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, used as a housekeeping gene) were designed with Primer Express Software (Applied Biosystems, Foster City, CA, USA) and supplied by Metabion (Martinsried, Germany).

Luciferase Activity. Firefly luciferase activity was measured using the luciferase assay reagent (Promega), and the results were normalized to the total cellular protein. The protein content was measured using the Coomassie blue method.

ELISA. IL-6 and MCP-1 concentrations in the culture medium were measured by enzyme-linked immunosorbent assay (ELISA) using reagents from R&D Systems Europe Ltd. (Abingdon, UK).

Carrageenan-Induced Paw Edema in the Mouse. The anti-inflammatory effects were studied by measuring carrageenan-induced paw edema in male C57BL/6 mice (Harlan Laboratories BV, Venray, The Netherlands). The study was carried out in accordance with the legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU) and approved by the National Animal Experiment Board (license ESLH-2009-07700/Ym-23). Animals were housed under conditions of optimum light, temperature, and humidity (12:12 h light−dark cycle, 22 ± 1 °C, 50−60%) with food and water provided ad libitum. Male mice were divided into six groups: control group, L-NIL group (50 mg/kg), pinosylvin group (100 mg/kg), monomethylpinosylvin group (100 mg/kg), resveratrol group (100 mg/kg), and dexamethasone group (2 mg/kg). Doses were selected on the basis of our preliminary experiments and data found in the literature.[17,18] Mice were dosed by intraperitoneal injection with 150 μL of normal saline or the tested compound at 2 h before carrageenan was applied. The mice were anesthesized by intraperitoneal injection of 0.5 mg/kg of medetomidine (Domitor 1 mg/mL, Orion Oyj, Espoo, Finland) and 75 mg/kg of ketamine (Ketalar 10 mg/mL, Pfizer Oy Animal Health, Helsinki, Finland), and thereafter the mice received a 30 μL intradermal injection into one hind paw of normal saline containing λ-carrageenan 1.5% (w/v). The contralateral paw received 30 μL of saline, and it was used as a control. Edema was measured 6 h after carrageenan injection by using a plethysmometer (Ugo Basile, Comerio, Italy). Carrageenan-induced edema is expressed as the difference in the volume change between the carrageenan-treated paw and the control paw.

Statistics. Results are expressed as the mean ± standard error of mean (SEM). Statistical significance of the results was calculated by one-way ANOVA with Dunnett's post test (dose curves) or Bonferroni's post test (multiple comparisons) by using GraphPad InStat 3 for Windows XP (Graph-Pad Software, San Diego, CA, USA). Differences were considered significant at * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$. EC_{50} values were calculated with GraphPadPrism 6 for Windows (Graph-Pad Software).

RESULTS

Effects of *P. sylvestris* Knotwood Extract, Pinosylvin, and Monomethylpinosylvin on iNOS Protein Expression and NO Production. Neither NO production nor iNOS expression was detectable in resting J774 macrophages. When the cells were activated through the Toll-like receptor 4 (TLR4) pathway by exposure to bacterial lipopolysaccharide (LPS, 10 ng/mL), both iNOS expression and NO production were significantly enhanced. The *P. sylvestris* knotwood extract inhibited iNOS protein expression and NO production in a dose-dependent manner (Figure 2), and about 50% inhibition was achieved at a concentration of 3 μg/mL.

P. sylvestris knotwood extract is rich in stilbenes particularly pinosylvin [5% (w/w)] and monomethylpinosylvin [12% (w/w)], and we continued by investigating the effects of these compounds on iNOS expression and NO production. Both pinosylvin and monomethylpinosylvin inhibited iNOS ex-

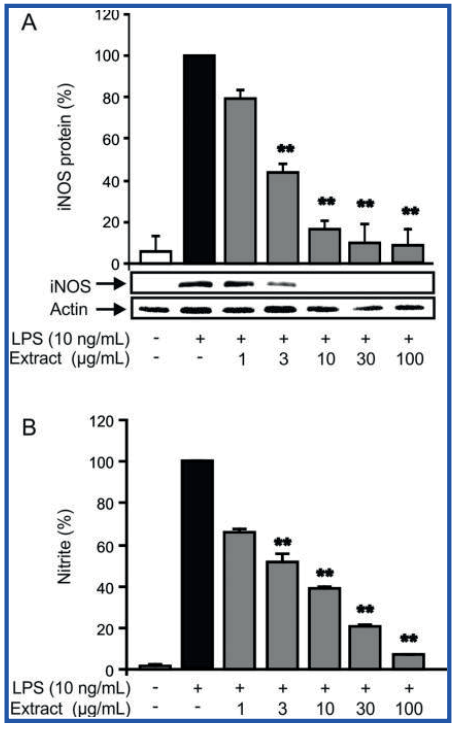

Figure 2. Effects of the *Pinus sylvestris* knot extract on LPS-induced (A) iNOS protein expression and (B) NO production in J774 macrophages as measured after a 24 h incubation. iNOS expression was measured by Western blot, and NO production was determined by measuring its metabolite nitrite in the culture medium by the Griess reaction. Values are expressed as the mean ± SEM, $n = 4$; (**) $p < 0.01$ as compared to cells incubated with LPS only.

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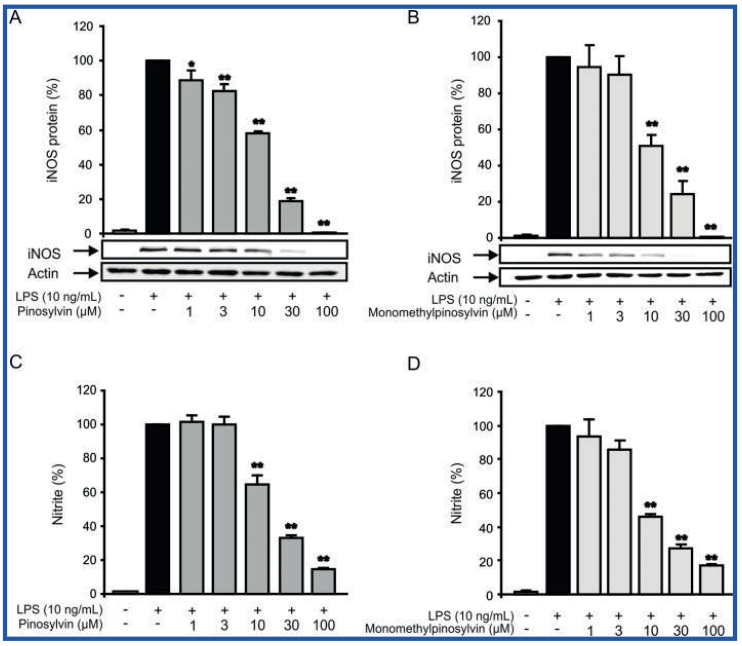

Figure 3. Effects of pinosylvin (A, C) and monomethylpinosylvin (B, D) on LPS-induced (A, B) iNOS protein expression and (C, D) NO production in J774 macrophages as measured after 24 h of incubation. iNOS expression was measured by Western blot, and NO production was determined by measuring its metabolite, nitrite, in the culture medium by the Griess reaction. Values are expressed as mean ± SEM, $n = 4$; (*) $p < 0.05$ and (**) $p < 0.01$ as compared to cells incubated with LPS only.

pression and NO production in a dose-dependent manner (Figure 3). Pinosylvin decreased iNOS expression with an EC_{50} value of 15 μM and monomethylpinosylvin with an EC_{50} value of 12 μM, whereas the EC_{50} values for inhibition of NO production were 13 μM for pinosylvin and 8 μM for monomethylpinosylvin. The effect was comparable to that of a well-known anti-inflammatory drug, dexamethasone, which decreased NO production by about 65% at a concentration of 10 μM.

After demonstrating that the stilbene components of the extract decreased iNOS expression and NO production, we also tested the effects of three other naturally occurring stilbene derivatives on iNOS protein expression and NO production: resveratrol as a positive control (this compound has been extensively evaluated in recent years) and astringin and isorhapontin, which have also been identified in extracts from Nordic conifers.[19] Resveratrol inhibited iNOS expression (EC_{50} = 18 μM) and NO production (EC_{50} = 6 μM) in activated macrophages in a dose-dependent manner (Figure 4), but the two other stilbenes (astringin and isorhapontin) were ineffective.

Effects of *P. sylvestris* Knotwood Extract, Pinosylvin, and Monomethylpinosylvin on iNOS mRNA Levels. In subsequent studies, we used quantitative RT-PCR to investigate the effects of *P. sylvestris* extract, pinosylvin, monomethylpinosylvin, and resveratrol (which was used as a control compound) on iNOS mRNA expression. J774 cells were incubated with the studied compounds for 2 h before LPS was added to activate the cells, and then mRNA levels were measured after a 6 h incubation with LPS. This time point was chosen according to the time curve of iNOS mRNA, where the maximal iNOS mRNA levels were detected at 6 h after the addition of LPS. *P. sylvestris* extract (100 μg/mL) and all tested stilbenes (100 μM) significantly lowered mRNA levels by 51−79% as seen in Figure 5.

Effects of *P. sylvestris* Knotwood Extract, Pinosylvin, and Monomethylpinosylvin on NF-κB-Mediated Transcription. To study in more detail their mechanisms of action, we investigated the effects of *P. sylvestris* extract, pinosylvin, and monomethylpinosylvin on NF-κB-mediated transcription because NF-κB is an important transcription factor for the iNOS gene. We used human embryonic kidney 293 cells that had been genetically modified to express luciferase (LUC) gene under the control of an NF-κB response element containing five NF-κB binding sites. *P. sylvestris* extract, pinosylvin, monomethylpinosylvin, and resveratrol (which was used as a control compound) significantly inhibited NF-κB-mediated transcription (measured as luciferase activity) (Figure 6). MG-132, a known NF-κB inhibitor, was also included in the experiments, and it reduced LUC-activity by >90%.

Effects of *P. sylvestris* Knotwood Extract, Pinosylvin, and Monomethylpinosylvin on Heme Oxygenase-1 Expression. HO-1 is a factor known to regulate NF-κB activation in macrophages.[20,21] Interestingly, the knotwood extract and the two purified stilbenes, pinosylvin and monomethylpinosylvin, as well as resveratrol all enhanced the HO-1 mRNA and protein levels in macrophages exposed to LPS (Figure 7). The HO-1 inducer hemin was used as a control compound.

Effects of *P. sylvestris* Knotwood Extract, Pinosylvin, and Monomethylpinosylvin on MCP-1 and IL-6 Production. We investigated whether *P. sylvestris* extract and the

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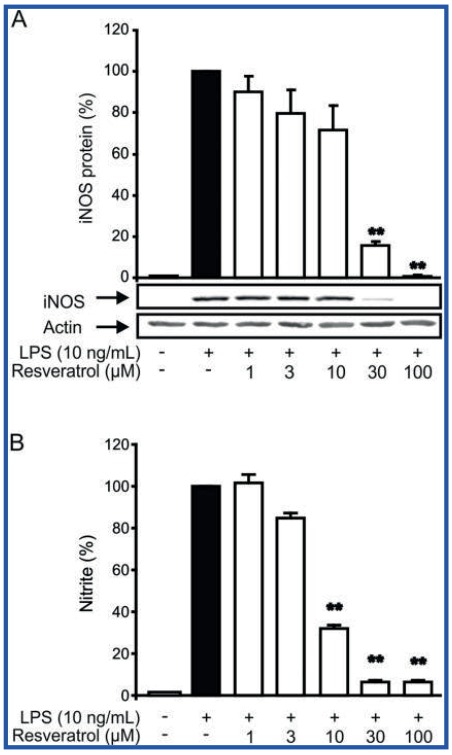

Figure 4. Effects of resveratrol on LPS-induced (A) iNOS protein expression and (B) NO production in J774 macrophages as measured after 24 h of incubation. iNOS expression was measured by Western blot, and NO production was assayed by measuring its metabolite, nitrite, in the culture medium by the Griess reaction. Values are expressed as mean ± SEM, $n = 4$; (**) $p < 0.01$ as compared to cells incubated with LPS only.

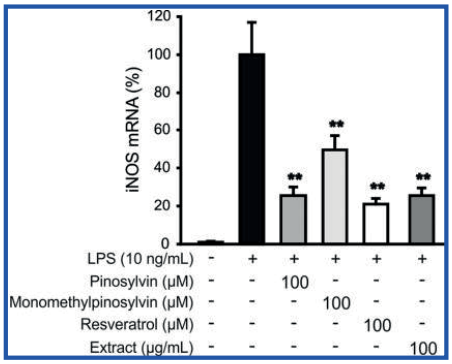

Figure 5. Effects of pinosylvin, monomethylpinosylvin, resveratrol, and the *P. sylvestris* knot extract on iNOS mRNA expression. J774 macrophages were stimulated with LPS in the presence or in the absence of the compounds and the extract for 6 h, and iNOS mRNA expression was measured by quantitative RT-PCR. The results were normalized against GAPDH mRNA and are expressed as mean ± SEM, $n = 4$; (**)$p < 0.01$ as compared to cells incubated with LPS only.

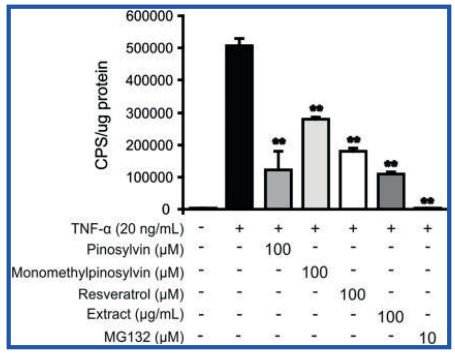

Figure 6. Effects of pinosylvin, monomethylpinosylvin, resveratrol, and the *P. sylvestris* knot extract on NF-κB-dependent transcription in HEK-293 cells transfected with luciferase reporter construct. HEK-pNF-κB(*luc2P*)hygro cells were stimulated with TNFα and treated with the extract or the compounds of interest or with the known NF-κB inhibitor MG132 for 5 h, and luciferase activity was measured. Results are expressed as mean ± SEM, $n = 4$; (**) $p < 0.01$ as compared to cells incubated with TNFα only.

stilbenes isolated from the extract would exert effects on other important inflammatory mediators by determining if they could modify the productions of the chemokine MCP-1 (Figure 8) and the pro-inflammatory cytokine IL-6 (Figure 9) in activated J774 macrophages. The extract of *P. sylvestris* reduced MCP-1 production in a dose-dependent manner with an EC_{50} value of 11 μg/mL. Furthermore, also pinosylvin (EC_{50} = 35 μM), monomethylpinosylvin (EC_{50} = 35 μM), and resveratrol (EC_{50} = 19 μM) reduced MCP-1 production. *P. sylvestris* extract (EC_{50} = 26 μg/mL) and monomethylpinosylvin (EC_{50} = 25 μM) had a moderate dose-dependent inhibitory effect also on IL-6 production. Similarly, resveratrol reduced IL-6 production with an EC_{50} value of 21 μM. Pinosylvin evoked about 30% inhibition on IL-6 production at a concentration of 30 μM. We also tested the effects of *P. sylvestris* extract, pinosylvin, and monomethylpinosylvin on COX-2 levels in activated macrophages, but none of the compounds had any inhibitory effect on the expression levels of COX-2 protein.

Effects of Pinosylvin and Monomethylpinosylvin on Carrageenan-Induced Paw Edema in the Mouse. Because both pinosylvin and monomethylpinosylvin displayed anti-inflammatory effects in the in vitro studies, we decided to test if they could also exhibit in vivo anti-inflammatory properties by using a carrageenan-induced paw inflammation model in the mouse. Intraperitoneal administration of known anti-inflammatory compounds, that is, the iNOS inhibitor L-NIL (50 mg/kg) and the glucocorticoid dexamethasone (2 mg/kg), reduced carrageenan-induced paw edema by >80%. Interestingly, also pinosylvin (100 mg/kg) and monomethylpinosylvin (100 mg/kg) decreased carrageenan-induced inflammation by almost 80%, whereas the inhibitory effect of resveratrol (100 mg/kg; used as a control compound) was only 50% (Figure 10).

DISCUSSION

The present study investigated the anti-inflammatory properties of knotwood extract of *P. sylvestris* and two of its stilbene constituents, pinosylvin and monomethylpinosylvin. We found that the knotwood extract of *P. sylvestris* inhibited LPS-induced NO production in activated macrophages in a dose-dependent

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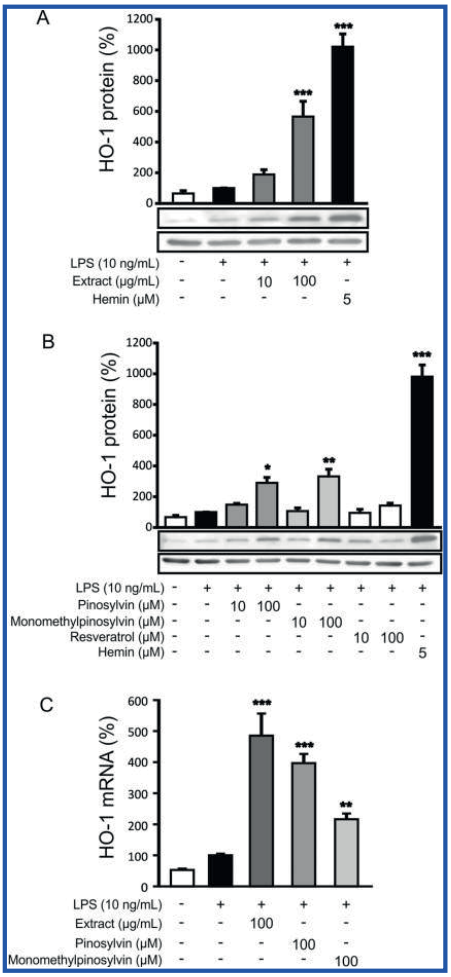

Figure 7. Effects of pinosylvin, monomethylpinosylvin, resveratrol, and the *P. sylvestris* knot extract on HO-1 protein expression (A, B) and mRNA expression (C). J774 macrophages were stimulated with LPS in the presence or in the absence of the compounds or the extract for 6 h, and HO-1 protein expression was measured by Western Blot or for 4 h and HO-1 mRNA expression was measured by quantitative RT-PCR. The results were normalized against GAPDH mRNA. The values are expressed as mean ± SEM, $n = 4$; (**) $p < 0.01$ as compared to cells incubated with LPS only.

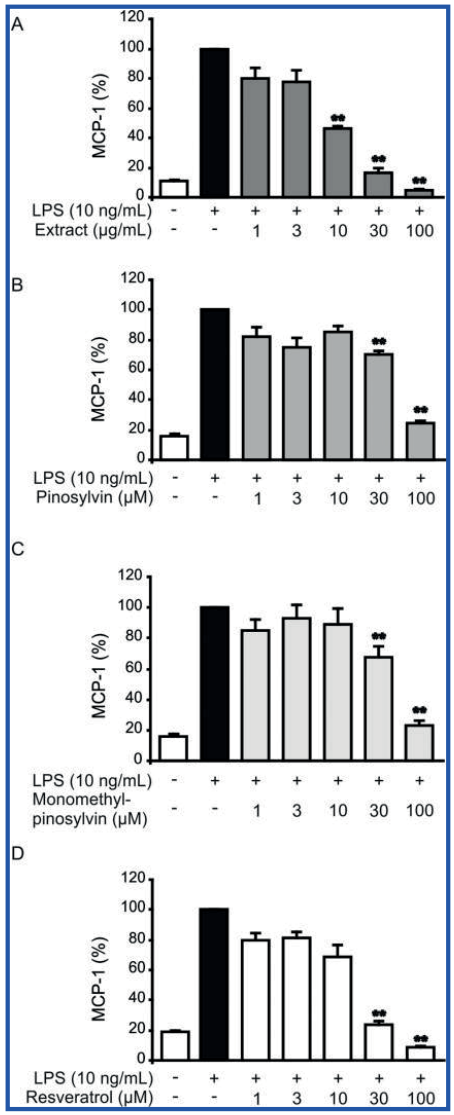

Figure 8. Effects of the *P. sylvestris* knot extract (A), pinosylvin (B), monomethylpinosylvin (C), and resveratrol (D) on MCP-1 production. J774 macrophages were stimulated with LPS for 24 h and thereafter the incubations were terminated and MCP-1 production was determined by ELISA. Results are expressed as mean ± SEM, $n = 4$; (**) $p < 0.01$ as compared to cells incubated with LPS only.

manner. This was attributable to the inhibitory effect on iNOS expression in response to inflammatory stimuli as both the iNOS protein and the mRNA levels were decreased. In addition, a clear reduction was observed in the expression of inflammatory genes IL-6 and MCP-1. Importantly, the anti-inflammatory effects of pinosylvin and monomethylpinosylvin were also found in vivo in a standard carrageenan-induced inflammatory paw edema model.

The immunomodulatory effects of *P. sylvestris* knot have not been reported earlier. It has been shown previously that the extract of *P. sylvestris* leaf buds could reduce NO production and iNOS mRNA expression in LPS and IFN-γ-stimulated murine macrophages but exerted no effects on COX-2 expression or PGE_2 production.[22] Karonen et al. have also reported that pine bark extracts possess anti-inflammatory properties,[23] but in these previous studies, the active constituents were not identified.

The knotwood extract was found to contain 5% (w/w) of pinosylvin and 12% of monomethylpinosylvin. The effects of these two stilbene derivatives on NO production and iNOS

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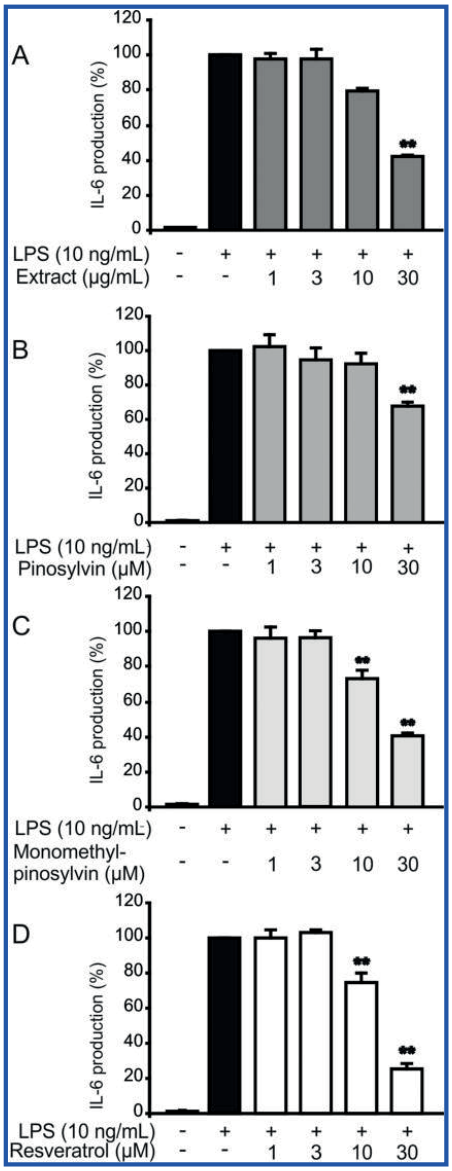

Figure 9. Effects of the *P. sylvestris* knot extract (A), pinosylvin (B), monomethylpinosylvin (C), and resveratrol (D) on IL-6 production. J774 macrophages were stimulated with LPS for 24 h, and thereafter the incubations were terminated and IL-6 production was determined by ELISA. Results are expressed as mean ± SEM, $n = 4$; (**) $p < 0.01$ as compared to cells incubated with LPS only.

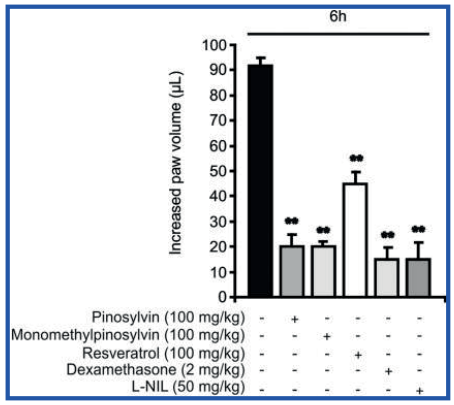

Figure 10. Effects of pinosylvin, monomethylpinosylvin, and resveratrol on carrageenan-induced paw inflammation in the mouse. Pinosylvin, monomethylpinosylvin, resveratrol, and control compounds L-NIL and dexamethasone were administered ip 2 h prior to carrageenan (1.5%) being injected into the paw. The contralateral paw was injected with the solvent and served as the control. Paw edema was measured before and 6 h after the carrageenan injection. Edema is expressed as the differences in the volume change between the carrageenan treated paw and the control paw. Results are expressed as mean ± SEM, $n = 6$; (**) $p < 0.01$.

expression were qualitatively similar to the effects of the extract, and thus it is likely that they account for the anti-inflammatory effects observed with the extract. Therefore, we continued the experiments with pinosylvin and monomethylpinosylvin, but we also included a well-known stilbene, resveratrol, as a control compound. In addition, two other stilbenes present in trees, astringin and isorhapontin, were also investigated.

Previously it has been shown that resveratrol could inhibit LPS-induced NO production in macrophages,[24−28] a result confirmed also in the present study. Furthermore, this effect was found to be shared by pinosylvin and monomethylpinosylvin. The present pinosylvin result is in line with the previous report from Park et al.[29] Some other natural substances from the stilbenoid group, namely, *cis*-mulberroside A, rhapontigenin, and piceatannol, have also been demonstrated to be able to decrease iNOS expression.[24,25,30] There are a few published studies on the structure−activity relationship of stilbenes on LPS-induced NO production which suggest that the structural balance between functional oxygen groups on the benzene rings is important for the biological activity.[24] In the previous study, the presence of a glucoside moiety was also found to reduce anti-inflammatory activity.[25] Here, the ineffectiveness of astringin and isorhapontin as compared to the other tested stilbenes may be explained by the fact that they are glycosylated forms and are perhaps poorly transported into the cell. It was decided to include astringin and isorhapontin in the present study because these compounds have also been reported to be present in Nordic conifers in addition to pinosylvin and monomethylpinosylvin.[19] There were no major differences between the potencies of pinosylvin, monomethylpinosylvin, and resveratrol on NO production or iNOS expression.

No previous data on the anti-inflammatory effects of monomethylpinosylvin have been reported, and there are only a few earlier reports on the corresponding properties of pinosylvin. Pinosylvin inhibited LPS-induced expression of pro-inflammatory cytokines IL-8 and TNF-α in THP-1 cells via the NF-κB signaling pathway.[31] Another study revealed that pinosylvin could reduce prostaglandin E_2 production in activated RAW 264.7 cells.[32]

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The present study extends the previous data by showing that the extract of *P. sylvestris* and also pinosylvin and monomethylpinosylvin were able to inhibit the iNOS-NO pathway in activated macrophages. The compounds suppressed also iNOS mRNA levels, which proves that a transcriptional mechanism is behind the inhibitory effect on iNOS expression. NF-κB is one of the key transcription factors regulating iNOS transcription. In the present study, we found that NF-κB-mediated transcription was inhibited by the extract of *P. sylvestris* and its two stilbene constituents, pinosylvin and monomethylpinosylvin, which may, at least partly, explain their inhibitory effects on iNOS and other NF-κB-dependent genes.

HO-1 has been shown to play a role in cellular and tissue defense against oxidative stress, possessing potent anti-inflammatory and cytoprotective properties.[33] In the present study, we found that the knot extract and its active stilbenes increased HO-1 expression in activated macrophages. This may represent a putative mechanism for their anti-inflammatory action and explain how they were able to decrease the NF-κB activity and reduce inflammatory gene expression. Some other compounds of natural origin have been reported to affect HO-1.[33] There is also one study reporting that resveratrol could induce HO-1 in human aortic smooth muscle cells, which supports our original findings on the protective effects of pinosylvin and monomethylpinosylvin through HO-1 expression.[34]

Additionally, it was important to determine whether our findings in vitro could be translated into an anti-inflammatory effect in vivo. We chose to test the effects of pinosylvin and monomethylpinosylvin in carrageenan-induced paw inflammation, which is a widely used experimental model of acute inflammation in preclinical pharmacological studies. The carrageenan-induced inflammatory response in the paw has been reported to be mediated partly by increased NO production because it is sensitive to the anti-inflammatory properties of iNOS inhibitors.[35,36] In the current study, the iNOS inhibitor, L-NIL, also reduced significantly carrageenan-induced acute inflammation. The inhibitory responses of pinosylvin and monomethylpinosylvin were comparable to that of L-NIL and another control compound, dexamethasone, which is a very efficacious anti-inflammatory drug that also inhibits iNOS expression.[37] In the present study, resveratrol was observed to inhibit carrageenan-induced paw edema, although this effect was not detected in the previous study of Gentilli et al.[38] The difference in the dosing of resveratrol in these studies may explain the different results. Gentilli and co-workers injected resveratrol shortly before the carrageenan challenge, whereas we administered the drug 2 h before carrageenan. However, it is noteworthy that pinosylvin and monomethylpinosylvin were more potent than resveratrol in combatting carrageenan-induced paw inflammation. As far as we are aware, there is only one previous study in which pinosylvin has been tested in an inflammatory model in vivo, in adjuvant arthritis in the rat.[18] The results support our present findings that pinosylvin possesses a significant anti-inflammatory action also in vivo.

In conclusion, the most interesting novel finding in this study was that pinosylvin and monomethylpinosylvin were found to have anti-inflammatory properties in vivo, possibly via a mechanism involving the stimulation of HO-1 levels leading to inhibition of NF-κB activity and ultimately to the suppression of inflammatory gene expression. These findings extend our understanding of the potential beneficial properties of naturally occurring stilbenes and may provide new insights in the development of novel anti-inflammatory treatments. In addition, the immunomodulatory potential of pinosylvin and its derivatives as well as their detailed mechanisms of action should be evaluated in further studies.

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ABBREVIATIONS USED

COX-2, cyclooxygenase-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HO-1, heme oxygenase 1; IFNγ, interferon-γ; iNOS, inducible nitric oxide synthase; IL-6, interleukin-6; L-NIL, L-N^6-(1-iminoethyl)lysine hydrochloride; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; NF-κB, nuclear factor kappa B; NO, nitric oxide; NOS, nitric oxide synthase; PGE_2, prostaglandin E_2; XTT, sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate

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PUBLICATION II

Anti-inflammatory Effects of Nortrachelogenin in Murine J774 Macrophages and in Carrageenan-Induced Paw Edema Model in the Mouse

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Anti-inflammatory effects of nortrachelogenin in murine J774 macrophages and in carrageenan-induced paw edema model in the mouse

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Abstract

Nortrachelogenin is a pharmacologically active lignan found in knot extracts of *Pinus sylvestris*. In previous studies, some lignans have been shown to have anti-inflammatory properties which made nortrachelogenin an interesting candidate for our study. In inflammation, bacterial products and cytokines induce the expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1). These enzymes synthesize factors which, together with proinflammatory cytokines, are important mediators and drug targets in inflammatory diseases.

The effects of nortrachelogenin on the expression of iNOS, COX-2 and mPGES-1 as well as on the production of nitric oxide (NO), prostaglandin E_2 (PGE_2) and cytokines IL-6 and MCP-1 were investigated in murine J774 macrophage cell line. In addition, we examined the effect of nortrachelogenin on carrageenan-induced paw inflammation in mice.

Interestingly, nortrachelogenin reduced carrageenan-induced paw inflammation in mice and inhibited the production of inflammatory factors NO, PGE_2, IL-6 and MCP-1 in J774 macrophages *in vitro*. Nortrachelogenin inhibited mPGES-1 protein expression but had no effect on COX-2 protein levels. Nortrachelogenin had also a clear inhibitory effect on iNOS protein expression but none on iNOS mRNA levels, and the proteasome inhibitor lactacystin reversed the effect of nortrachelogenin on iNOS expression suggesting a post-transcriptional mechanism of action. The results revealed hitherto unknown anti-inflammatory properties of nortrachelogenin which could be utilized in the development of anti-inflammatory treatments.

Keywords: nortrachelogenin, lignan, iNOS, inflammation, *Pinus sylvestris,* Pinaceae

Abbreviations: COX-2, cyclooxygenase-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase; IL-6, interleukin-6; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; mPGES-1, microsomal prostaglandin E synthase-1; NO, nitric oxide; NOS, nitric oxide synthase; PGE_2, prostaglandin E_2; XTT, sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate

Introduction

Inflammation is a complex and tightly regulated reaction to injurious, irritating or pathogenic factors. Its purpose is to eliminate these harmful factors and to induce the regenerative processes to repair the tissue damage. In chronic inflammatory diseases or autoimmune disorders, the inappropriate, prolonged and/or poorly coordinated inflammatory response results in the symptoms and signs observed in patients. Excessively increased activation of inflammatory and immune cells leads to over-production of inflammatory cytokines and other factors involved in inflammation which cause harm to the host and, therefore, serve as powerful targets for anti-inflammatory treatments.[1]

Nitric oxide (NO) and prostaglandins (PGs) play an important role in the generation of the inflammatory response as well as in regulating several physiological responses including vascular tone and blood clotting [2,3]. Their biosynthesis is significantly increased in inflamed tissue, and they contribute to the development of the cardinal signs of acute inflammation.

The first PGE_2 synthase, namely mPGES-1, was identified 1999 by Jakobsson et al [4]. It is induced by many proinflammatory cytokines and it seems to have a role in the pathophysiology of several diseases. mPGES-1 is a potential new target for the drug development especially for inflammatory diseases. [5]

Nitric oxide (NO) is a small gaseous signalling molecule which acts as a regulatory and effector molecule in inflammation. NO is produced by three different forms of NOS, namely neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). iNOS expression is induced in inflammatory and tissue cells in response to bacterial products and inflammatory cytokines, and it is responsible for prolonged production of NO in high concentrations [2]. In animal studies iNOS inhibitors have had

beneficial effects in several models of acute and chronic inflammation [6]. However, the results in clinical studies have not been so promising [7]. Nevertheless, it is evident that NO plays an immunoregulatory role in the induction and resolution of inflammation in a concentration dependent manner, and it is likely that NO is functionally relevant to host defence. [7-9]

Interleukin-6 (IL-6) is a 184 amino acid glycosylated protein that mediates inflammation, immune response and hematopoiesis via specific receptor, IL-6R. Because of wide range of biological activities and pathological role of IL-6 in several diseases, targeting IL-6 in drug development has become important. Tocilizumab, a humanized anti-IL-6R monoclonal antibody is in the market for the treatment of rheumatoid arthritis in more than 100 countries. [10] Besides cytokines also chemokines are released in the early phase of inflammation. Monocyte chemoattractant protein-1 (MCP-1) is a central chemokine in the inflammatory response that serves as a chemoattractant for monocytes and macrophages, and plays key roles in many immune processes. [11]

The lignans are a group of polyphenolic compounds found in plants. They are basically formed by oxidative coupling of two phenylpropane units. Coniferous trees present a rich source of lignans. [12] Considerable health benefits have been implicated with lignan-rich diet, including prevention of cancers and cardiovascular diseases [13,14]. In previous studies, some lignans have shown to have anti-inflammatory properties [15] which made nortrachelogenin (Fig. 1) an interesting candidate for our study.

In our preliminary search of bioactive compounds from knot extracts of *Pinus sylvestris*, the anti-inflammatory compound nortrachelogenin was identified. Nortrachelogenin is a pharmacologically active lignan which was first isolated from *Wikstroemia indica* in 1979 by Kato et al [16]. In addition, it is found in several other resources from nature like *Carissa spinarum, Daphne oleoides, Juniperus*

rigida and *Trachelospermum jasminoides* [17-20]. According to our knowledge, only a limited amount of data is available on the biological or pharmacological effects of nortrachelogenin *in vitro* and *in vivo*. In previous studies, nortrachelogenin has been shown to have anti-plasmoidial activity *in vitro* and antileukemic properties *in vivo* but it did not inhibit the growth of the DMBA-induced mammary tumors in rats [21-23]. It was also moderately active against HIV-1 *in vitro* [24]. In a recent study, nortrachelogenin was shown to enhance tumor necrosis factor related apoptosis-inducing ligand and to inhibit Akt signalling [25] but its anti-inflammatory potential remains practically unexplored.

In the presence study, we investigated the anti-inflammatory properties of nortrachelogenin in activated macrophages *in vitro* by measuring its effects on the expression of inflammatory enzymes iNOS, COX-2 and mPGES-1 and on the production of inflammatory mediators NO, PGE_2, IL-6 and MCP-1. Further, we were interested if the anti-inflammatory properties found in those *in vitro* studies are also translated to the *in vivo* situation and measured the effects of nortrachelogenin on carrageenan-induced paw inflammation in the mouse.

Results

NO production and iNOS expression were not detectable in resting J774 macrophages. When the cells were activated through Toll-like receptor 4 (TLR4) pathway by exposing them to bacterial lipopolysaccharide (LPS), iNOS expression and NO production were significantly enhanced. Nortrachelogenin decreased iNOS protein expression and NO production in a dose-dependent manner (Fig. 2A,B). Reduction of iNOS protein levels was about 50 % at 1 μM concentration and over 90 % at 30 μM concentration (EC50 value 1 μM). Nortrachelogenin inhibited also NO production but the effect was smaller: 49 % inhibition was found when nortrachelogenin was given to the cells at 30 μM concentration. The effect was not stimulus-specific as nortrachelogenin (30 μM) also inhibited NO production induced by interferon gamma (IFN-γ) alone or in combination with interleukin-1beta (IL-1β) and tumor necrosis factor alpha (TNF-α) ($p<0.01$) (Fig. 2C,D).

Next, we examined the effects of nortrachelogenin on iNOS mRNA levels. J774 cells were cultured with LPS in the presence and absence of nortrachelogenin and mRNA levels at time points 3 h, 6 h, 12 h and 24 h were measured. The maximal iNOS mRNA levels were found following 6 h incubation, and thereafter the mRNA levels decreased rapidly. Nortrachelogenin had no effect on iNOS mRNA levels at any time point measured (Fig. 3) suggesting that the effect of nortrachelogenin on iNOS protein expression (and subsequent NO production) is mediated trough post-transcriptional mechanisms.

There is previous evidence showing that iNOS protein is degraded by the proteasome pathway and some pharmacological compounds enhance that effect [26]. Therefore we investigated the effect of nortrachelogenin on LPS-induced iNOS expression in the presence of the proteasome inhibitor

lactacystin [27,28]. Lactacystin was added to the cells after 8 h incubation with LPS or with the combination of LPS and nortrachelogenin. As expected, iNOS protein levels measured after 24 h incubation were higher in the cells treated with LPS and lactacystin than in cells treated with LPS only (Fig. 4). Interestingly, nortrachelogenin had no effect on iNOS protein levels in LPS treated macrophages in the presence of lactacystin while it significantly inhibited iNOS expression in the absence of the proteasome inhibitor. The findings sustained our hypothesis that nortrachelogenin is likely to inhibit iNOS expression and inducible NO production by enhancing iNOS protein degradation through proteasome.

To find out whether nortrachelogenin has effects on other important inflammatory mediators we investigated its effects on the production of chemokine MCP-1 and proinflammatory cytokine IL-6 (Fig. 5) in J774 macrophages activated with LPS. Nortrachelogenin reduced both MCP-1 and IL-6 production in a dose-dependent manner with EC50 values 7 μM for MCP-1 and 25 μM for IL-6, respectively. The highest concentration used (30 μM) caused about 60 % inhibition on MCP-1 and 55 % inhibition on IL-6 production.

We also studied the effects of nortrachelogenin on the expression of COX-2 and mPGES-1 in LPS-stimulated J774 macrophages. Interestingly, nortrachelogenin reduced mPGES-1 protein levels and, accordingly, inhibited the synthesis of its product PGE_2 (EC50 values 14 μM for mPGES-1 and 17 μM for PGE_2). However, nortrachelogenin had no effect on COX-2 expression (Fig. 6).

As nortrachelogenin proved to have anti-inflammatory effects *in vitro*, we wanted to investigate if those effects are also translated to *in vivo* and studied the effects of nortrachelogenin in carrageenan-induced paw inflammation in the mouse. Intraperitoneal administration of nortrachelogenin (100 mg/kg) reduced carrageenan-induced paw edema at 3 h by 53 % and at 6 h by 50 % as seen in Figure

7, whereas the known anti-inflammatory glucocorticoid dexamethasone (2 mg/kg) decreased carrageenan-induced paw inflammation by about 80 %.

Discussion

In the present study, we evaluated anti-inflammatory effects of nortrachelogenin, a lignan ingredient found in knot extracts of *Pinus sylvestris*. The results showed that nortrachelogenin suppressed iNOS expression and NO production by enhancing iNOS protein degradation through proteasome pathway. Nortrachelogenin decreased also the production of pro-inflammatory factors PGE_2, IL-6 and MCP-1 in J774 macrophage cell line. More importantly, nortrachelogenin inhibited significantly carrageenan-induced paw edema in the mouse. To our knowledge this is the first report showing that nortrachelogenin has anti-inflammatory effects; and they were evident both *in vitro* and *in vivo*.

Carrageenan-induced paw edema is a commonly used model in inflammation research representing features of acute inflammation and innate immunity. Carrageenan-induced inflammation is mediated by inflammatory cells especially macrophages and neutrophils. The development of edema is described as a biphasic event [29]. The later phase (3-6 h after carrageenan injection) is strongly associated with increased expression of iNOS and COX-2 and local production of nitric oxide and prostaglandins. During the later phase IL-6, IL-1β, TNF-α and MCP-1 levels are also enhanced [30,31]. Recently it has been shown that also transient receptor potential ankyrin 1 (TRPA1) mediates carrageenan-induced inflammation as carrageenan-induced response was found to be attenuated in TRPA1 deficient mice. [32]

Nortrachelogenin decreased carrageenan-induced paw edema possibly by reducing cytokine formation and by down-regulating iNOS / NO and mPGES-1 / PGE_2 pathways as was shown in the macrophage cell model. The response of nortrachelogenin was parallel to that of the control compound dexamethasone which is a very efficacious anti-inflammatory drug that also inhibits iNOS expression [33]; however, dexamethasone was more potent than nortrachelogenin and it was used at a clearly lower dose. Interestingly, also selective iNOS inhibitors have been shown to be very effective in carrageenan-induced paw edema model [34]. Nortrachelogenin also inhibited MCP-1 production. This could lead to decreased amount of inflammatory cells to be recruited to the inflammation site.

Most known iNOS suppressing compounds regulate iNOS expression at the transcriptional level but some compounds, e.g. PPARα agonists, natural compound curcumin and lignan compound arctigenin have been reported to promote degradation of iNOS protein through proteasome pathway [26,35,36]. In the present study, nortrachelogenin had no effect on iNOS mRNA levels but, interestingly, the proteasome inhibitor lactacystin reversed the effect of nortrachelogenin on iNOS protein expression. Accordingly, when measured at the same time point (after 24h incubation), nortrachelogenin had a greater inhibitory effect on iNOS protein levels than on nitrite levels (the latter reflecting the cumulative NO production during the entire incubation). These data together support the idea that nortrachelogenin enhances iNOS protein degradation through proteasome pathway leading to reduced iNOS levels and suppressed NO production. Additional studies are needed to clarify in further detail which proteasome subcomponents are targets of nortrachelogenin. At least 26S and 20S have been reported to be important for the degradation of iNOS [27,28].

Lignans are plant polyphenols traditionally classified into two types, classical lignans and neolignans. Classical lignans are formed from two phenylpropanes linked in a β-β' (8–8') fashion, while neolignans are those dimers whose coupling patterns differ from β-β'linkage. Nortrachelogenin is a classical lignan related to enterolactone also grouped to dibenzylbutyrolactone lignans. [37] Formerly it has been shown that nortrachelogenin has a moderate inhibitory effect on TNF-α production in peripheral blood stimulated with LPS but in the same study the effect on IL-1 biosynthesis was inconsistent [19]. Bis-5,5-nortrachelogenin has also been shown to inhibit NO production in RAW 264.7 murine macrophage-like cell line [38]. The present study extends the previous knowledge by showing that nortrachelogenin has anti-inflammatory properties *in vivo* and inhibits iNOS and mPGES-1 expression and NO, PGE_2, IL-6 and MCP-1 production in activated macrophages *in vitro*. In a previous study of During et al. [39] pinoresinol had strongest anti-inflammatory properties of six studied lignans i.e. secoisolariciresinoldiglucoside, secoisolariciresinol, pinoresinol, lariciresinol, matairesinol and hydroxymatairesinol; pinoresinol was found to reduce IL-6 and MCP-1 production in Caco-2 cells stimulated with IL-1β with IC50 values of 12.5 μM and 100 μM, respectively [39]. Those findings support the results of our study, even though pinoresinol has a different furofuran structure than nortrachelogenin and it is known to convert to enterolactone in the colon [40].

In conclusion, we showed here, for the first time, that nortrachelogenin has anti-inflammatory properties *in vitro* and *in vivo* by down-regulating inflammatory gene expression in macrophages and by attenuating the carrageenan-induced paw edema in the mouse, likely through multiple independent or cross-talking mechanisms. Nortrachelogenin is a promising new anti-inflammatory compound for interfering iNOS and mPGES-1 expression and cytokine production in various inflammatory conditions.

Materials and Methods

Materials

Nortrachelogenin (purity > 95%) was purchased from Arbonova and dexamethasone (purity > 97%) from Sigma Chemical Co. Rabbit polyclonal iNOS (sc-650), COX-2 (sc-1745) and β-actin (sc-1615-R) antibodies and HPR-conjugated goat polyclonal anti-rabbit antibody and donkey polyclonal anti-goat antibody were purchased from Santa Cruz Biotechnology Inc and rabbit polyclonal mPGES-1 (AS03031) from Agrisera. All other reagents were from Sigma Chemical Co unless otherwise stated.

Cell culture

Murine J774 macrophages (American Type Culture Collection) were cultured at 37° C in 5% CO_2 atmosphere and grown in Dulbecco's Modified Eagle's Medium (DMEM) with glutamax-I containing 10 % heat-inactivated foetal bovine serum, penicillin (100 units/mL), streptomycin (100 µg/mL) and amphotericin B (250 ng/mL) (Invitrogen). Cells were seeded on 96 well plates for XTT-test, and on 24 well plates to measure NO, PGE_2, MCP-1 and IL-6 production or iNOS, mPGES-1 and COX-2 expression. Cell monolayers were grown for 72 h to confluence before the experiments were started and the compounds of interest were added in fresh culture medium. Cytotoxicity of nortrachelogenin was ruled out by measuring cell viability using Cell Proliferation Kit II (Roche Diagnostics).

Nitrite Assays

NO production was determined by measuring the accumulation of nitrite, a stable metabolite of NO in aqueous milieu, by Griess reaction [41].

Western Blot Analysis

At the indicated time points, cells were rapidly washed with ice-cold phosphate-buffered saline (PBS) and solubilized in cold lysis buffer containing 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 1% Triton-X-100, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodiumorthovanadate, 20 µg/mL leupeptin, 50 µg/mL aprotinin, 5 mM sodium fluoride, 2 mM sodium pyrophosphate and 10 µM *n*-octyl-β-D-glucopyranoside. After incubation for 15 min on ice, lysates were centrifuged (12,000 g, 4° C for 10 min), and supernatants were collected and stored in SDS sample buffer at -20° C. An aliquot of the supernatant was used to determine protein concentration by the Coomassie blue method [42].

Protein samples (20 µg of lysates) were analyzed according to standard Western blotting protocol as described previously [43]. The membrane was incubated with the primary antibody in the blocking solution overnight at 4º C, and with the secondary antibody in the blocking solution for 1 h at room temperature. Bound antibody was detected using SuperSignal West Pico or Dura chemiluminescent substrate (Pierce) and Image Quant LAS 4000 mini imaging system (GE Healthcare Bio-Sciences AB). The quantitation of the chemiluminescent signal was carried out with the use of Imaging Quant TL software (GE Healthcare Bio-Sciences AB).

RNA extraction and quantitative RT-PCR

Primers and probes for quantitative reverse transcription polymerase chain reaction (RT-PCR) were obtained from Metabion International AG. At the indicated time points, culture medium was removed

and total RNA was extracted with GenElut Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) according to the manufacturer's instructions and as previously described [44]. Total RNA was reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems). cDNA obtained from the RT-reaction was diluted 1:20 with RNAse-free water and subjected to PCR using TaqMan Universal PCR Master Mix and ABI PRISM 7000 Sequence detection system (Applied Biosystems). The primers and probes were the following: mouse iNOS forward 5'-CCTGGTACGGGCATTGCT-3' (300 nM), mouse iNOS reverse 5'-GCTCATGCGGCCTCCTT-3' (300 nM), mouse iNOS probe 5'-CAGCAGCGGCTCCATGACTCCC-3' (150 nM), mouse GAPDH forward 5'-GCATGGCCTTCCGTGTTC-3' (300 nM), mouse GAPDH reverse 5'-GATGTCATCATACTTGGCAGGTTT-3' (300 nM), mouse GAPDH probe 5'-TCGTGGATCTGACGTGCCGCC-3' (150 nM). The primer and probe sequences and concentrations were optimized according to the manufacturer's guidelines in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C. PCR reaction parameters were as follows: incubation at 50º C for 2 min, incubation at 95º C for 10 min, and thereafter 40 cycles of denaturation at 95º C for 15 s and annealing and extension at 60º C for 1 min. Each sample was determined in duplicate. A standard curve method was used to determine the relative mRNA levels.

Enzyme-linked immunosorbent assay

IL-6, MCP-1 and PGE_2 were measured in the culture medium by enzyme linked immunosorbent assay (ELISA) using reagents from R&D Systems Europe Ltd (IL-6 and MCP-1) and Cayman Chemicals (PGE_2).

Carrageenan-induced paw edema in mice

Anti-inflammatory effects were studied by measuring carrageenan-induced paw edema in male C57BL/6 mice (Harlan Laboratories BV). The study was carried out in accordance with the legislation for the protection of animals used for scientific purposes (directive 2010/63/EU) and The Finnish Act on Animal Experimentation (62/2006). The study was authorized in Finland by the national Animal Experiment Board, license number ESLH-2009-07700/Ym-23 (granted September 23, 2009). Paw edema was induced under anesthesia and all efforts were made to minimize suffering. Mice were housed under conditions of optimum light, temperature and humidity (12:12 h light:dark cycle, 22±1° C, 50-60 %) with food and water provided *ad libitum*. Male mice aged 10 weeks were divided into three groups: control group, nortrachelogenin (100 mg/kg) group and dexamethasone (2 mg/kg) group. The doses of nortrachelogenin and dexamethasone based on our preliminary experiments. Mice were dosed with 150 µL of PBS-10 % DMSO vehicle or the tested compound by intraperitoneal injection 2 h before carrageenan was applied. The mice were anesthesized by intraperitoneal injection of 0.5 mg/kg of medetomide (Domitor 1 mg/mL, Orion Oyj) and 75 mg/kg of ketamine (Ketalar 10 mg/mL, Pfizer Oy Animal Health), and thereafter the mice received 30 µL injection of sterile saline containing 1.5 % of λ-carrageenan (w/v) in one hind paw. The contralateral paw received 30 µL of saline and it was used as a control. Edema was measured before and 3 and 6 h after carrageenan injection with plethysmometer (Ugo Basile). Edema is expressed as the difference, in µL, between the volume changes of the carrageenan treated paw and the control paw.

Statistics

Results are expressed as the mean + standard diviation (SD). Statistical significance of the results was calculated by one-way ANOVA with Dunnett's post test (dose curves) or Bonferroni´s post test (multiple comparisons) by using GraphPad InStat 3 for Windows XP (GraphPad Software). Differences were considered significant at *$p < 0.05$, **$p<0.01$ and ***$p<0.001$. EC50 values were calculated with GraphPad Prism version 7.01 for Windows (GraphPad Software).

Conflict of interest statement

The authors declare no conflicts of interests.

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Figure legends

Fig. 1. Chemical structure of nortrachelogenin.

Fig. 2. Effects of nortrachelogenin on LPS induced iNOS protein expression and NO production in J774 macrophages. iNOS expression (A) was measured by western blot and NO production (B,C,D) as its stable metabolite nitrite by Griess reaction after 24 h incubation. Values are expressed as mean + SD, n=4, *$p<0.05$ and **$p<0.01$ as compared to cells cultured with LPS (A,B), IFN-γ (C) or cytomix (D). Cytomix is a combination of IFN-γ (10 ng/mL), TNF-α (20 ng/mL) and IL-1β (10 ng/mL).

Fig.3. Effects of nortrachelogenin on iNOS mRNA expression. J774 macrophages were cultured with LPS alone or with LPS and nortrachelogenin and RNA was extracted at time points 3 h, 6 h, 12 h and 24 h. iNOS mRNA expression was measured by quantitative RT-PCR. The results were normalised against GAPDH mRNA and are expressed as mean + SD, n=4.

Fig. 4. Effects of the proteasome inhibitor lactacystin and nortrachelogenin on iNOS expression in J774 macrophages. Cells were stimulated with LPS in the presence and in the absence of nortrachelogenin. After 8 h incubation the proteasome inhibitor lactacystin was added into the culture. Proteins were extracted after 24 h incubation and iNOS protein levels were measured by western blot. Values are expressed as mean + SD, n=4, **$p<0.01$ and ns=not significant as compared to cells cultured with LPS only.

Fig. 5. Effects of nortrachelogenin on MCP-1 (A) and IL-6 (B) production. J774 macrophages were stimulated with LPS in the presence of increasing concentrations of nortrachelogenin for 24 h

before the incubations were terminated and MCP-1 and IL-6 concentrations in the culture media were determined by ELISA. Results are expressed as mean + SD, n=4, *p<0.05 and **p<0.01 as compared to cells cultured with LPS only.

Fig. 6. Effects of nortrachelogenin on LPS-induced PGE_2 production and COX-2 and mPGES-1 expression. PGE_2 production (A), COX-2 protein expression (B) and mPGES-1 protein expression (C) were measured in J774 macrophages after 24 h incubation. COX-2 and mPGES-1 protein levels were measured by western blot and PGE_2 production by ELISA. Values are expressed as mean + SD, n=4, **p<0.01 as compared to cells cultured with LPS only.

Figure 7. Effects of nortrachelogenin and the anti-inflammatory steroid dexamethasone on carrageenan-induced paw inflammation model in the mouse. Nortrachelogenin (100 mg/kg) and dexamethasone (2 mg/kg) were administered i.p. 2 h prior to carrageenan (1.5 %) was injected into the paw. Paw edema was measured before, and 3 and 6 h after carrageenan injection with a plethysmometer. Edema is expressed as the difference in volume changes between the carrageenan treated paw and the contralateral vehicle-injected paw. Results are expressed as mean + SD, n=6, ***p<0.001 as compared to mice without drug treatment.

Fig.1

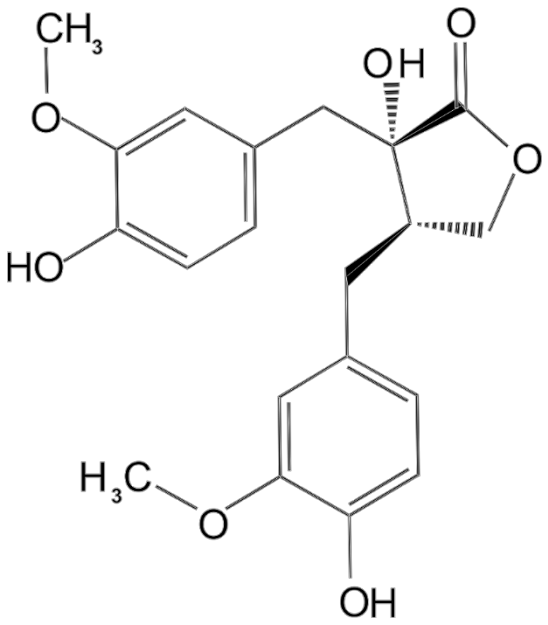

Fig. 2

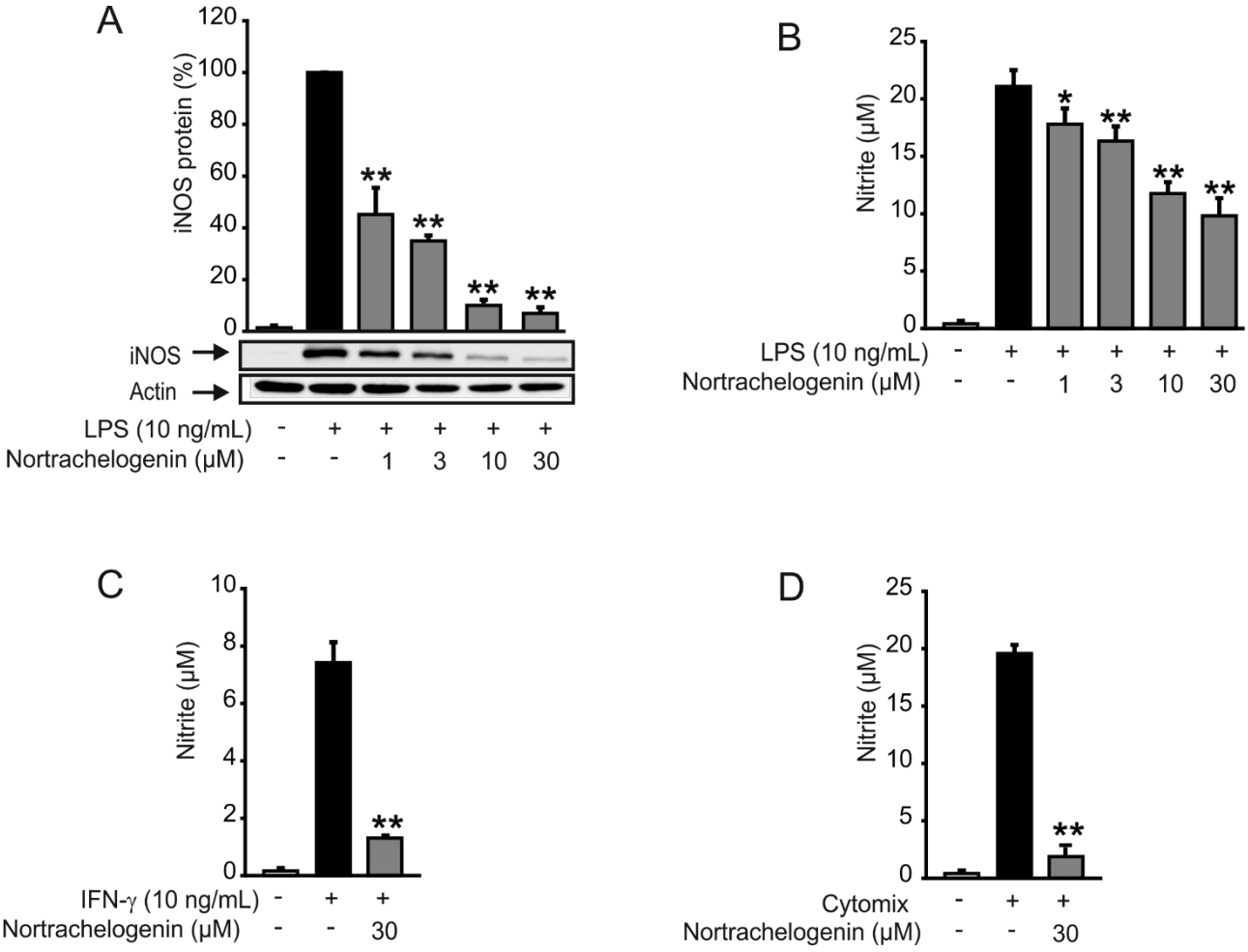
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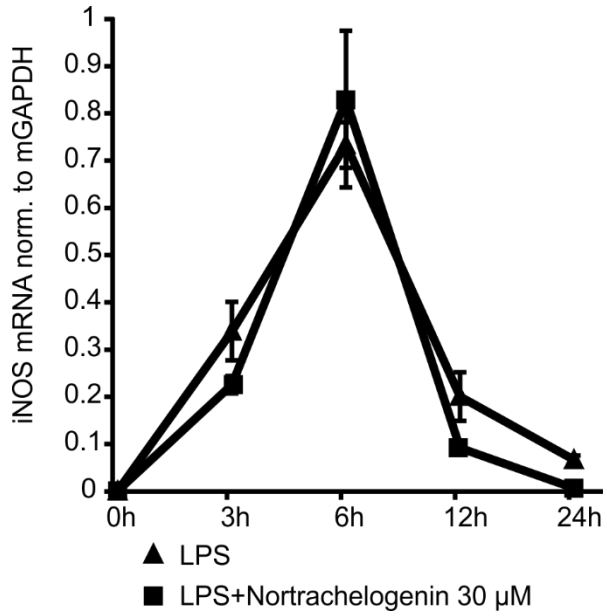
Fig. 3


iNOS mRNA norm. to mGAPDH
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12h
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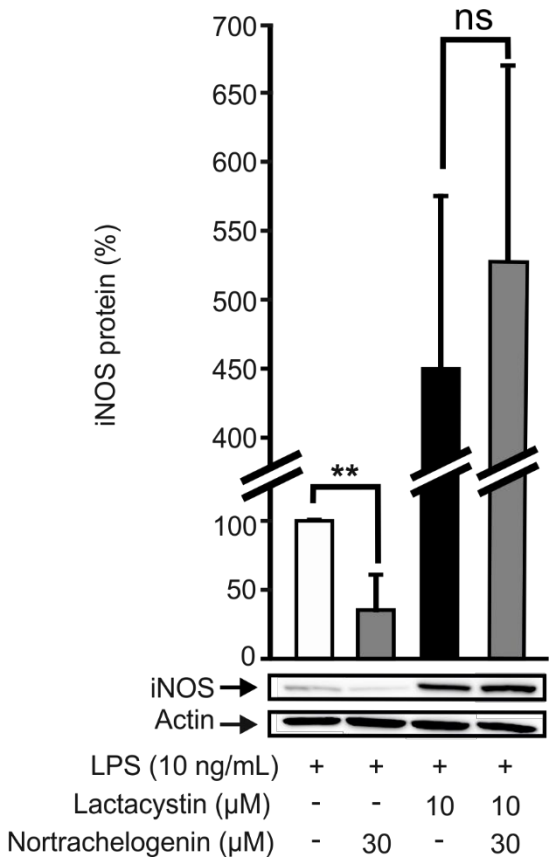
LPS
LPS+Nortrachelogenin 30 µM

Fig. 4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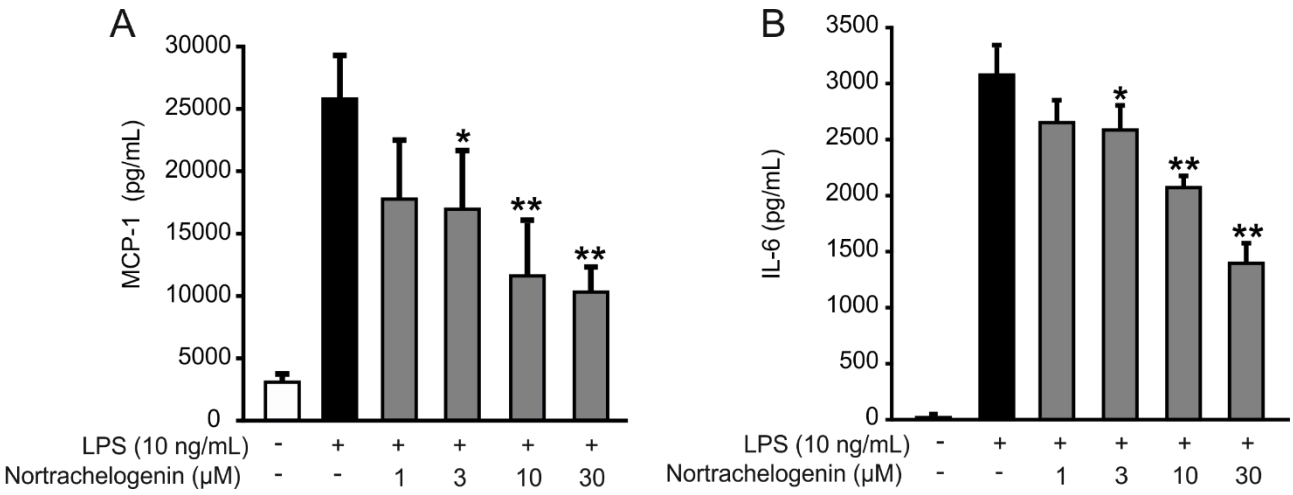
Nortrachelogenin (μM) - 30 - 30

Fig. 5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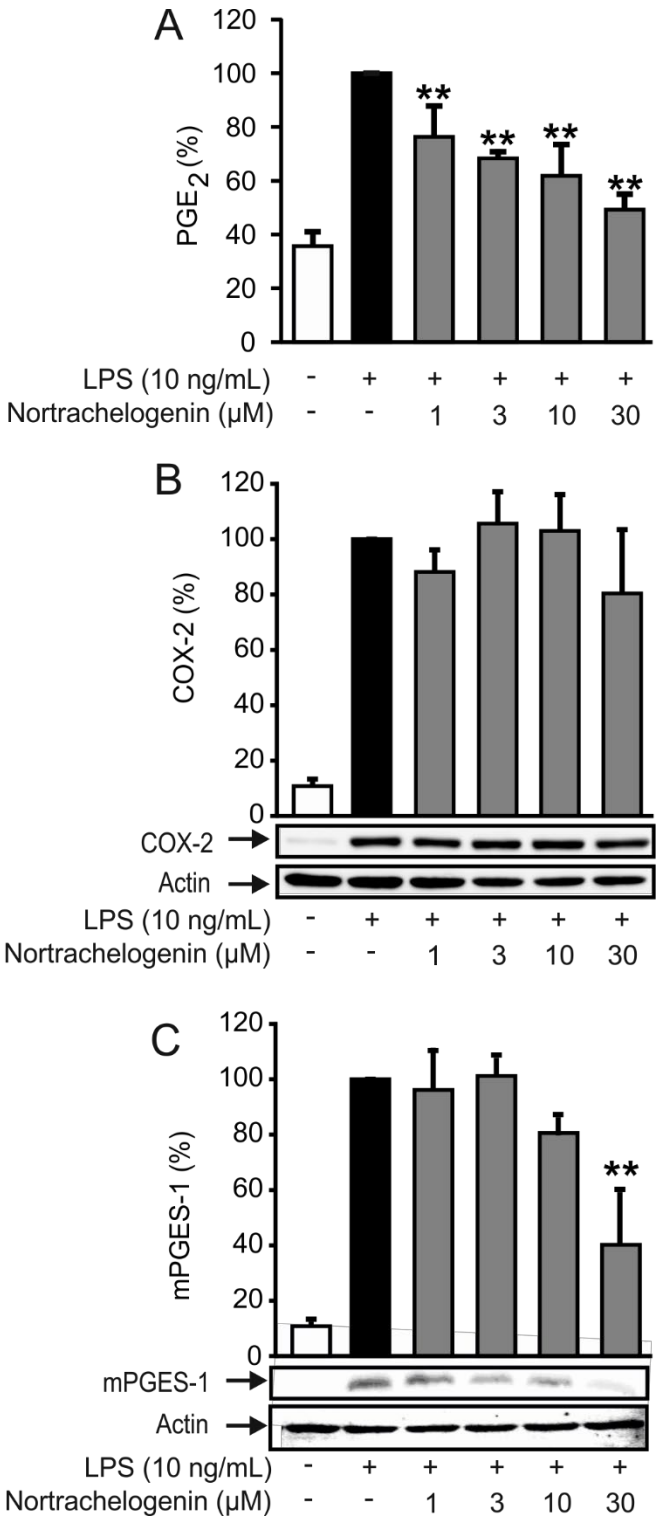
LPS (10 ng/mL) - + + + + +
Nortrachelogenin (μM) - - 1 3 10 30

Fig. 6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PGE2 (%)
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LPS (10 ng/mL) - + + + + +
Nortrachelogenin (μM) - - 1 3 10 30
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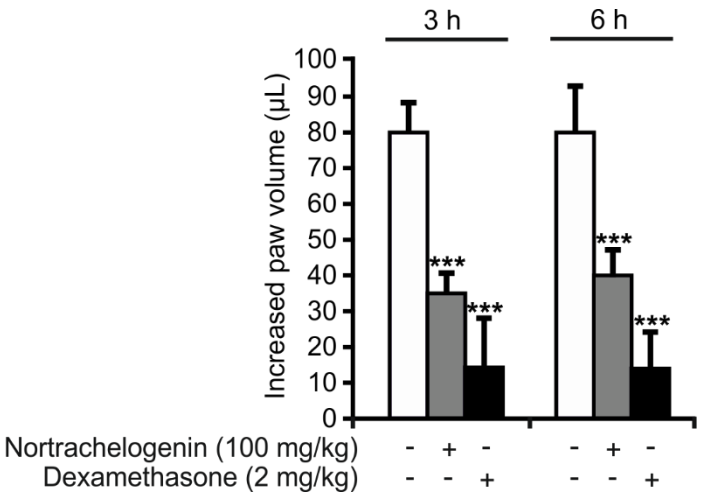
20
0
COX-2 (%)
COX-2
Actin
LPS (10 ng/mL) - + + + + +
Nortrachelogenin (μM) - - 1 3 10 30
C
120
100
80
60
40
20
0
mPGES-1 (%)
**
mPGES-1
Actin
LPS (10 ng/mL) - + + + + +
Nortrachelogenin (μM) - - 1 3 10 30

Fig. 7


3 h
6 h
Increased paw volume (µL)
100
90
80
70
60
50
40
30
20
10
0
***
***
***
***
Nortrachelogenin (100 mg/kg) - + - - + -
Dexamethasone (2 mg/kg) - - + - - +

PUBLICATION III

Betulin Derivatives Effectively Suppress Inflammation *in Vitro* and *in Vivo*

Mirka Laavola, Raisa Haavikko, Mari Hämäläinen, Tiina Leppänen, Riina Nieminen, Sami Alakurtti, Vânia M. Moreira, Jari Yli-Kauhaluoma and Eeva Moilanen

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Article

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Betulin Derivatives Effectively Suppress Inflammation in Vitro and in Vivo

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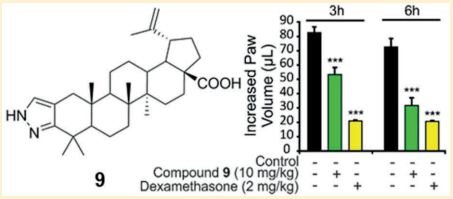
ABSTRACT: Betulin is a pharmacologically active triterpenoid found in the bark of the birch tree (*Betula* sp. L.). Betulin and betulinic acid are structurally related to anti-inflammatory steroids, but little is known about their potential anti-inflammatory properties. In the present study, the inflammatory gene expression and the anti-inflammatory properties of betulin, betulinic acid, and 16 semisynthetic betulin derivatives were investigated. Betulin derivatives **3**, **4**, and **5** selectively inhibited the expression of the inducible nitric oxide synthase (iNOS) in a post-transcriptional manner. They also inhibited nitric oxide (NO) production but had no effect on the other inflammatory factors studied. More interestingly, a new anti-inflammatory betulin derivative **9** with a wide-spectrum anti-inflammatory activity was discovered. Compound **9** was found to suppress the expression of cytokines interleukin-6 (IL-6) and monocyte chemotactic protein-1 (MCP-1), as well as that of prostaglandin synthase-2 (COX-2) in addition to iNOS. The in vivo anti-inflammatory effect of compound **9** was indicated via significant suppression of the carrageenan-induced paw inflammation in mice. The results show, for the first time, that the pyrazole-fused betulin derivative (**9**) and related compounds have anti-inflammatory properties that could be utilized in drug development.



Betulin (**1**) is a naturally occurring triterpenoid, and it is found in high amounts in the bark of the birch tree (*Betula* sp.L.). Betulin (**1**) has a wide range of biological activities, and it is easily converted into betulinic acid (**2**), which has typically shown to be more potent than betulin (**1**).[1] Betulin (**1**) and its semisynthetic derivatives have been reported to have anti-HIV, antimalarial, and antileishmanial properties.[2−4] Betulin and betulin-enriched birch bark extracts showed significant antiproliferative effects in several human cancer cell lines.[5] Betulinic acid (**2**) and some of its derivatives also exhibit antitumor activity against, for example, melanoma, skin epidermoid carcinoma, prostate cancer, and leukemia cells.[6−9] Both betulin (**1**) and betulinic acid (**2**) possess similarly antiangiogenic activity in vivo.[9,10] In addition, some data suggest that betulin (**1**) and/or betulinic acid (**2**) have anti-inflammatory properties as reported in the context of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and carrageenan-induced inflammation and in lipopolysaccharide (LPS) induced acute lung inflammation.[1,5,11−13] However, a thorough characterization of their anti-inflammatory properties has not been reported.

Inflammation is an adaptive response that is triggered by tissue injury or by agents such as microbial products during infection. Its purpose is to eliminate harmful factors and to induce the regenerative processes to repair tissue damage. However, inflammation is also at the pathogenic core of many diseases including arthritic, pulmonary, gastrointestinal, cardiovascular, and neurodegenerative diseases.[14] Inappropriate, prolonged, or poorly coordinated inflammatory response may result in tissue injury and chronic inflammatory disease. Increased activation of inflammatory and immune cells leads to overproduction of inflammatory factors such as pro-inflammatory cytokines, chemokines, and enzymes.[15] Down-regulation of these inflammatory factors is beneficial in inflammatory diseases and many anti-inflammatory drugs inhibit/down-regulate inflammatory enzymes or mediators. The identification of novel compounds that can effectively suppress excessive or aberrant inflammation can therefore significantly contribute toward finding improved therapeutic options for patients suffering from these conditions. The currently available anti-inflammatory drugs are not always optimal or useful as they are not effective in all patients or cause intolerable adverse effects, and in many cases they have not been proved to prevent the progression of disease.[16,17] Therefore, the search for novel anti-inflammatory substances is medically well justified.

Herein, the inflammatory gene expression and the anti-inflammatory properties of betulin (**1**), betulinic acid (**2**), and 16 semisynthetic betulin derivatives were investigated. The

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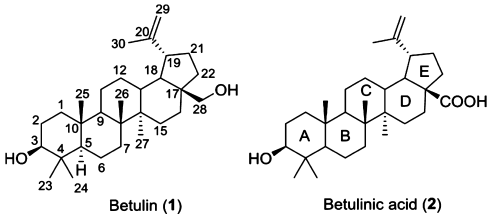
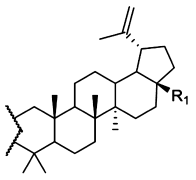
effects of these compounds on the expression of the inflammatory enzymes iNOS and COX-2 and the production of the proinflammatory cytokine IL-6, the chemotactic factor MCP-1, and the highly reactive inflammatory factor NO in activated macrophages are reported. The studies were complimented by evaluating the effects of the most promising compound in the well-characterized in vivo model widely used in pharmacological studies, namely, in the carrageenan-induced paw inflammation in mice.

RESULTS AND DISCUSSION

Chemistry. The structures of the semisynthetic compounds are shown in Figures 1 and 2. The synthesis of compounds **3**–**18** is described in detail elsewhere.[18−20]

Screening of the Effects of Betulin Derivatives on Inflammatory Gene Expression in Vitro. iNOS expression or NO production was not noticeable in resting murine J774 macrophages. When the cells were activated through the Toll-like receptor 4 (TLR4) pathway by exposing them to bacterial lipopolysaccharide (LPS), iNOS expression and NO production were significantly enhanced. Sixteen betulin derivatives, betulin (**1**), and betulinic acid (**2**) were tested and 11 of them caused more than 50% inhibition of iNOS protein expression when used at 10 μM concentrations, as measured by Western blot analysis (Table 1). Compounds **3**, **4** and **5** reduced iNOS protein expression by more than 90% at 10 μM concentration, and were selected for further dose−response effect studies. The effect of these three compounds was comparable to that of the highly effective anti-inflammatory agent, the glucocorticoid dexamethasone, when used at similar concentration (10 μM). Thirteen of the tested compounds also inhibited NO production in a statistically significant manner when used at 10 μM concentration (Table 1). The betulin derivatives **3**, **4**, **6**, and **7**, and betulin (**1**) (at 10 μM concentration) reduced NO production by more than 50%, and the control compound dexamethasone (10 μM) inhibited NO production by 85%. These findings are in agreement and extend those of Costa et al., who reported that betulinic acid (**2**) inhibited LPS-induced NO production in murine peritoneal macrophages.[21]





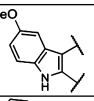
ID	Ring A	R_1	ID	Ring A	R_1
3			11		COOH
4		COOH	12		$CONH_2$
5		$CONH_2$	13		COOH
6		COOH	14		COOH
9		COOH	17		COOH
10			18		COOH

Figure 1. Structures of betulin and betulinic acid derivatives.

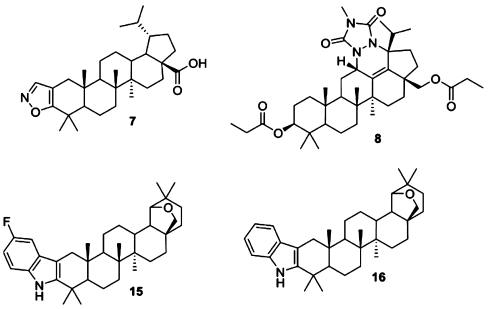


Figure 2. Structures of compounds **7**, **8**, **15**, and **16**.

The effects of the betulin derivatives on the expression of another significant inflammatory enzyme, namely, COX-2, in activated macrophages were also studied. Only compounds **8**–**10** reduced COX-2 protein expression in a statistically significant manner at 10 μM concentration showing about 40−60% reduction as detected in Western blot analysis (Table 1). Compound **8** was the most potent betulin derivative to suppress COX-2 protein expression levels by 57%, whereas about 90% down-regulation was achieved with control compound dexamethasone (10 μM).

IL-6 is one of the most important pro-inflammatory cytokines in innate and adaptive immunity and its antagonists have shown beneficial effects and are in clinical use to treat rheumatoid arthritis. Therefore, the effects of this series of compounds were also studied on the expression of IL-6 in activated macrophages. Compounds **2**, **7**, **8**, **9**, and **13**, when used at 10 μM concentrations, decreased IL-6 production in a statistically significant manner (Table 1). Substantial reduction of more than 60% was seen only with compound **9**. Inhibition by the control compound, dexamethasone (10 μM) was 86%.

MCP-1 is an inducible chemokine that recruits inflammatory cells into the sites of inflammation. Compounds **5**, **7**, **9**, **13**, and **14** (at 10 μM) reduced MCP-1 production in activated J774 macrophages in a statistically significant manner showing more than 25% inhibition (Table 1). Clearly, the most effective compound was again compound **9** with greater than 70%

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Table 1. Effects of Betulin Derivatives on NO Production and on iNOS, COX-2, IL-6, and MCP-1 Expression in J774 Macrophages

betulin derivative (10 μM)	NO (%)	iNOS (%)	COX-2 (%)	IL-6 (%)	MCP-1 (%)
LPS control	100	100	100	100	100
1	47.9 ± 4.6 ***	63.4 ± 5.3 ***	175.1 ± 18.0	133.9 ± 5.6	206.5 ± 6.5
2	69.0 ± 0.8 ***	57.6 ± 5.8 ***	96.0 ± 7.8	66.1 ± 5.4 ***	98.5 ± 5.1
3	31.3 ± 2.3 ***	6.2 ± 1.0 ***	127.0 ± 4.5	98.3 ± 2.3	136.9 ± 5.2
4	41.7 ± 2.9 ***	8.3 ± 0.5 ***	144.1 ± 14.3	100.1 ± 5.0	138.4 ± 4.5
5	66.5 ± 4.2 ***	8.3 ± 1.0 ***	105.5 ± 4.8	90.9 ± 6.2	57.6 ± 2.0 ***
6	45.3 ± 1.6 ***	38.3 ± 4.7 ***	107.1 ± 8.3	87.1 ± 2.5	118.0 ± 4.7
7	40.5 ± 2.4 ***	14.5 ± 4.0 ***	88.5 ± 10.4	69.2 ± 3.0 **	65.3 ± 3.6 **
8	55.7 ± 8.2 ***	50.5 ± 4.6 ***	43.0 ± 6.3 ***	60.0 ± 8.8 ***	80.3 ± 6.9
9	53.3 ± 4.0 ***	27.5 ± 2.5 ***	58.2 ± 4.3 ***	39.5 ± 5.4 **	28.0 ± 2.5 ***
10	113.1 ± 6.7	57.5 ± 10.8 ***	49.6 ± 5.3 ***	91.4 ± 8.3	102.1 ± 11.8
11	58.9 ± 1.9 ***	12.1 ± 3.2 ***	104.6 ± 6.1	119.1 ± 3.0	93.6 ± 8.3
12	111.9 ± 5.6	34.9 ± 2.3 ***	89.9 ± 5.7	82.8 ± 5.4	79.4 ± 6.9
13	71.4 ± 1.8 ***	63.6 ± 7.9 ***	93.4 ± 3.5	70.7 ± 6.1 **	68.3 ± 6.5 **
14	97.5 ± 1.7	95.1 ± 3.8	162.6 ± 18.1	123.5 ± 5.9	73.1 ± 3.4 *
15	122.6 ± 3.0	83.9 ± 7.1	91.8 ± 13.8	99.3 ± 4.1	108.5 ± 4.7
16	96.5 ± 0.7	94.5 ± 9.4	94.8 ± 11.4	92.1 ± 2.0	96.2 ± 1.8
17	79.5 ± 1.4 **	66.6 ± 5.6 **	140.9 ± 7.9	108.9 ± 3.3	83.4 ± 2.0
18	75.2 ± 2.9***	40.7 ± 7.1 ***	117.4 ± 7.2	131.7 ± 7.7	87.0 ± 3.8

Values are expressed as mean ± SEM, $n = 4$, * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$ as compared to cells cultured with LPS only.

inhibition when reduction by the control compound, dexamethasone (10 μM), was 38%.

Dose–Response Effects and Effects on mRNA Levels. All compounds that decreased iNOS protein levels over 90% in the first screening experiments at 10 μM concentrations were selected for more detailed dose–response studies. Clear dose–response effects were seen with compounds **3**, **4**, and **5** on iNOS protein expression, and the IC_{50} values ranged between 0.3 and 3 μM (Figure 3).

Next, the effects of these three compounds on iNOS mRNA levels were measured. Activated J774 cells were cultured with compounds **3**, **4**, and **5** at a concentration of 10 μM and iNOS mRNA levels were measured following 6 h incubation. The time point was chosen on the basis of the time curve of iNOS mRNA, where the iNOS mRNA levels peak at 6 h. None of the compounds had any significant effect on iNOS mRNA levels (Figure 4), suggesting that the effect of betulin derivatives on iNOS protein expression and subsequent NO production could be mediated trough post-transcriptional regulation of iNOS expression.

In the primary screening experiments, compound **9** had a statistically significant inhibitory effect on all of the inflammatory factors measured. The dose–response studies were carried out for the factors that were inhibited over 50% by compound **9** at a concentration of 10 μM. The dose–response curves at concentrations of 0.3–10 μM are shown in Figures 5 and 6. Because compound **9** significantly down-regulated iNOS, IL-6, and MCP-1 protein expression, its effects on mRNA levels of those inflammatory factors were measured (Figure 7). Activated J774 cells were incubated with compound **9** at a concentration of 10 μM and mRNA levels were measured at three time points, namely, 3, 6, and 24 h. Compound **9** suppressed iNOS mRNA levels significantly at 6 and 24 h, IL-6 mRNA at 6 h, and MCP-1 mRNA at 3 and 6 h.

Acute Inflammation in Vivo. Compound **9** proved to have important anti-inflammatory effects in vitro, and therefore, it was selected for in vivo testing. Interestingly, the anti-inflammatory in vitro effects were also translated to in vivo, and compound **9** decreased carrageenan-induced paw inflammation in mice in a statistically significant manner. Intraperitoneal administration of compound **9** at a dose of 10 mg/kg reduced carrageenan-induced paw edema at 3 h by 27% and at 6 h by 44%, as shown in Figure 8. The control compound, dexamethasone (2 mg/kg), decreased carrageenan-induced paw inflammation by about 64% at 6 h.

Structure–Activity Relationships. When measuring iNOS expression in activated macrophages, the natural compounds betulin (**1**) and betulinic acid (**2**) showed a moderate inhibition (37 and 42%, respectively), whereas betulonic acid (**6**) showed higher (62%) inhibition (Table 1). Modifying the C-28 hydroxymethyl group of betulin by conversion into an oxime moiety (**3**) improved the inhibitory effect on iNOS expression. Furthermore, fusing a heterocyclic group to the A-ring of betulonic acid (**6**) increased the inhibitory activity. The addition of a fused pyridine **11** or pyrazine **4** ring to the A ring of the betulinic acid (**2**) skeleton increased the inhibitory activity. On the other hand, changing the C-28 hydroxycarbonyl group to the primary amide group of the pyrazine derivative **12** resulted in decreased activity (65%). The isoxazole derivative of dihydrobetulonic acid (**7**) had an excellent activity, which was further improved (92%) by changing its hydroxycarbonyl group to the primary amide group and isopropyl to isopropenyl moiety at C-20 as in compound **5**. When the primary amide group of the compound **5** was changed to a formyl group of **10**, activity decreased to moderate levels. Pyrazole derivative **9** displayed good inhibitory activity. Compound **18** was active in suppressing iNOS expression, whereas its tentative metabolite **13** only showed moderate inhibitory activity. Only the indole derivatives of betulin and allobetulin showed weaker activity (5–33%) than betulin.

When investigating the effects on COX-2 expression, some of the compounds enhanced COX-2 expression. The betulin-fused heterocyclic compound **8** showed the best suppressive effect on

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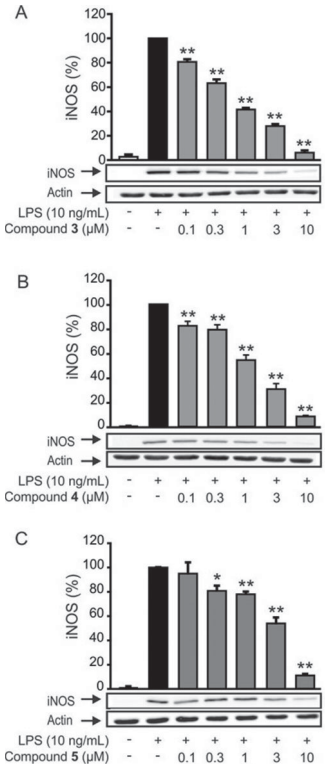

Figure 3. Effects of compounds **3**, **4**, and **5** on LPS-induced iNOS protein expression in J774 macrophages. iNOS expression was measured by Western blot after 24 h incubation with actin as the loading control. Values are expressed as mean + SEM, $n = 4$, * = $p < 0.05$, and ** = $p < 0.01$ as compared to cells cultured with LPS only.

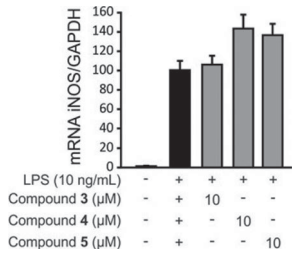

Figure 4. Effects of compounds **3**, **4**, and **5** on iNOS mRNA levels. J774 macrophages were cultured with LPS alone or with LPS and the compound of interest for 6 h and iNOS mRNA was measured by quantitative RT-PCR. The results were normalized against GAPDH mRNA and are expressed as mean + SEM, $n = 4$.

COX-2 expression (57%), two other active compounds being **10** (50%) and **9** (42%). Based on these results, no clear correlation between the structure of the derivatives and the suppressive effect on COX-2 expression could be found.

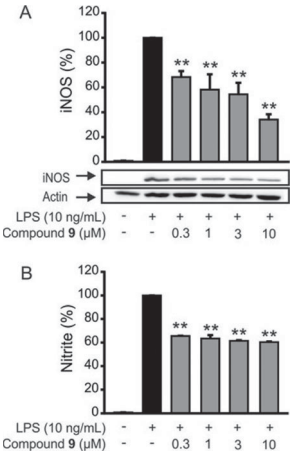

Figure 5. Effects of increasing concentrations of compound **9** on LPS-induced (A) iNOS protein expression and (B) NO production in J774 macrophages. iNOS expression was measured by Western blot after 24 h incubation with actin as the loading control and NO production as its stable metabolite nitrite by Griess reaction. Values are expressed as mean + SEM, $n = 4$, ** = $p < 0.01$ as compared to cells cultured with LPS only.

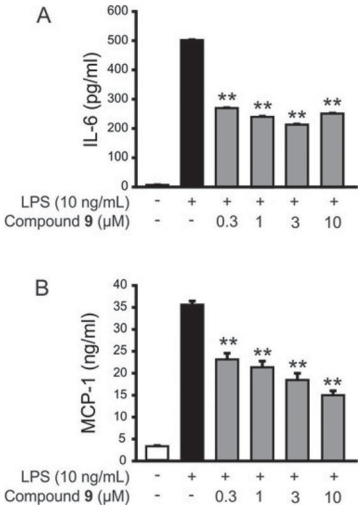

Figure 6. Effects of compound 9 on (A) IL-6 and (B) MCP-1 production. J774 macrophages were stimulated with LPS in the presence of increasing concentrations of compound **9** for 24 h before the incubations were terminated, and MCP-1 and IL-6 concentrations in the culture media were determined by ELISA. Results are expressed as mean + SEM, $n = 4$, ** = $p < 0.01$ as compared to cells cultured with LPS only.

Interestingly, compound **9** showed a good activity in all assays carried out (i.e., inhibition of iNOS, COX-2, IL-6, and MCP-1 expression).

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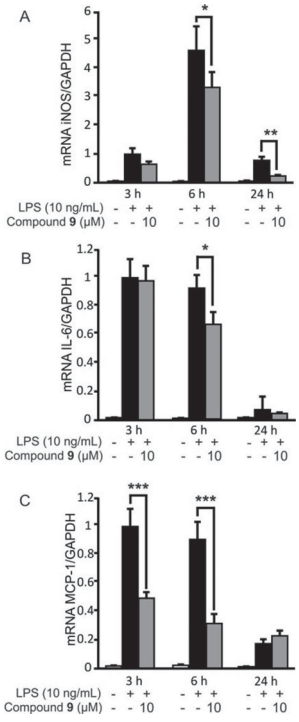

Figure 7. Effects of compound **9** on (A) iNOS, (B) IL-6, and (C) MCP-1 mRNA levels. J774 macrophages were cultured with LPS alone or with LPS and compound **9** for 3, 6, or 24 h, and thereafter total RNA was extracted. mRNA expression was measured by quantitative RT-PCR. The results were normalized against GAPDH mRNA and are expressed as mean + SEM, $n = 4$, * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$ as compared to cells cultured with LPS only.

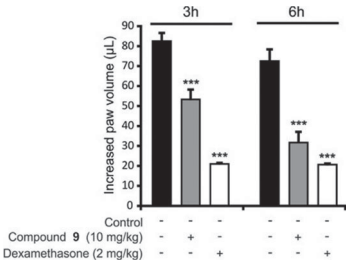

Figure 8. Effect of compound **9** on carrageenan-induced paw inflammation in the mouse. Compound **9** at the dose of 10 mg/kg or dexamethasone (2 mg/kg) or their vehicle was administered ip. two hours prior to carrageenan (1.5%) was injected into the paw. The inflammatory edema was measured 3 and 6 h after the carrageenan injection and was compared to the basal level. Results are expressed as mean + SEM, $n = 6–12$, *** = $p < 0.001$ as compared to mice without drug treatment. The contralateral control paw injected with saline developed no measurable edema.

Mechanisms Related to the Anti-Inflammatory Effects. The results showed that there were two types of anti-inflammatory compounds found among the betulin derivatives. Compounds **3**, **4**, and **5** showed a selective effect on iNOS expression. They suppressed iNOS protein levels and NO production but had no effect on iNOS mRNA levels, suggesting a post-transcriptional mechanism on iNOS expression. Many of the known compounds which down-regulate iNOS expression, target various transcriptional mechanisms. In addition, there is evidence that some compounds (e.g., PPARα agonists) and the natural compounds curcumin and arctigenin, promote the degradation of iNOS through the proteasome pathway.[22−24] Furthermore, dexamethasone inhibits iNOS expression and NO production by destabilizing its mRNA.[25] Other possible post-transcriptional mechanisms could be a specific class of noncoding RNAs, microRNAs (miRNAs). miRNAs are short nucleotides that are complementary to 3′-UTR mRNA sequences and able to inhibit translation or to direct mRNA for degradation. Recent evidence points to an important role for miRNAs in the inflammatory process.[26] Some miRNAs have been identified that regulate also human and rodent iNOS gene expression.[27] miRNA-939 was reported to regulate iNOS expression in human hepatocytes.[28] miR-939 decreased cytokine induced hiNOS protein expression but had no effect on hiNOS mRNA levels or mRNA stability. The two other miRNAs reported to down-regulate iNOS gene expression are miR-146a (human and mouse) and miR-26a (human).[27] There are no anti-inflammatory drugs shown to inhibit iNOS through this kind of mechanism until now, but it is possible that small molecules directly modulate miRNAs leading to mRNA degradation.[29]

Compound **9** was found to suppress several inflammatory factors (i.e., iNOS, IL-6 and MCP-1 at protein and mRNA levels), suggesting interference with a transcriptional mechanism. NF-κB is one of the key transcription factors for iNOS and important for regulation of IL-6 and MCP-1 also. Other relevant transcription factors behind these inflammatory genes could be AP-1, IRF-1, SP-1, or STAT-1.[30−32] Interestingly, it was found that the anti-inflammatory effects of compound **9**, which were first discovered in vitro, were also translated to in vivo. Compound **9** decreased the carrageenan-induced paw inflammation in a statistically significant manner in mice, which is a widely used model for evaluating acute inflammation and in search of anti-inflammatory drugs. Carrageenan-induced inflammation is mediated by inflammatory cells especially macrophages and neutrophils. The development of edema is described as a biphasic event. The later phase is strongly associated with local production of NO and prostaglandins and increase of iNOS and COX-2 expression.[33] During the later phase, IL-6, IL-1β, and TNF-α levels are also enhanced.[34] Inhibition of MCP-1 production is also beneficial because it could lead to a decreased amount of inflammatory cells to be recruited to the inflammation site. Compound **9** was found to suppress iNOS and COX-2 expression as well as NO, IL-6, and MCP-1 production in macrophages in vitro, which effects may contribute to the anti-inflammatory effect of the compound **9** observed in the carrageenan-induced paw edema in vivo.

In conclusion, we showed that the betulin derivatives **3**, **4**, and **5** inhibited iNOS expression and NO production in activated macrophages. They are thus likely to have anti-inflammatory properties in disease states complicated with increased iNOS expression. In addition, a novel anti-inflammatory betulin derivative pyrazolobetulinic acid (**9**)

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Table 2. Primer and Probe Sequences

gene	oligonucleotide	sequence 5′→3′
murine iNOS	forward primer	CCTGGTACGGGCATTGCT
	reverse primer	GCTCATGCGGCCTCCTT
	probe	CAGCAGCGGCTCCATGACTCCC
murine IL-6	forward primer	TCGGAGGCTTAATTACACATGTTC
	reverse primer	CAAGTGCATCATCGTTGTTCATAC
	probe	CAGAATTGCCATTGCACAACTCTTTTCTCA
murine GAPDH	forward primer	GCATGGCCTTCCGTGTTC
	reverse primer	GATGTCATCATACTTGGCAGGTTT
	probe	TCGTGGATCTGACGTGCCGCC

with a wide-spectrum anti-inflammatory activity was identified. Pyrazolobetulinic acid (**9**) was found to suppress the expression of the inflammatory enzymes iNOS and COX-2 and of the pro-inflammatory cytokines IL-6 and MCP-1, in activated macrophages. The anti-inflammatory effect was also evident in vivo, as compound **9** significantly suppressed the carrageenan-induced paw inflammation in mice. The results suggest that betulin derivatives, particularly pyrazolobetulinic acid (**9**) and derivatives thereof, offer properties that could be utilized in anti-inflammatory drug development.

EXPERIMENTAL SECTION

General Experimental Procedures . Rabbit polyclonal iNOS (sc-650), COX-2 (sc-1745), and β-actin (sc-1615-R) antibodies, as well as HRP-conjugated goat polyclonal antirabbit antibody and donkey polyclonal antigoat antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). Other reagents were from Sigma Chemical Co (St. Louis, MO, U.S.A.) unless otherwise stated.

Cell Culture. Murine J774 macrophages (American Type Culture Collection, Rockville, MD, U.S.A.) were cultured at 37 °C in 5% CO_2 atmosphere and grown in Dulbecco's Modified Eagle's Medium (DMEM) with glutamax-I containing 10% heat-inactivated fetal bovine serum, penicillin (100 units/mL), streptomycin (100 μg/mL), and amphotericin B (250 ng/mL, Invitrogen, Paisley, U.K.). Cells were seeded on 96-well plates for XTT-test, and on 24-well plates to measure NO, MCP-1, and IL-6 production or iNOS and COX-2 expression. Cell monolayers were grown for 72 h to confluence before the experiments were started and the compounds of interest were added in fresh culture medium. Cytotoxicity of betulin derivatives was ruled out by measuring cell viability using Cell Proliferation Kit II (Roche Diagnostics, Mannheim, Germany).

Nitrite Assays. NO production was determined by measuring the accumulation of nitrite, a stable metabolite of NO in aqueous milieu, by the Griess reaction.[35]

Western Blot Analysis. At the indicated time points, cells were rapidly washed with ice-cold phosphate-buffered (pH 7.4) saline (PBS; 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl) and solubilized in cold lysis buffer containing 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 1% Triton-X-100, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 20 μg/mL leupeptin, 50 μg/mL aprotinin, 5 mM NaF, 2 mM sodium pyrophosphate, and 10 μM *n*-octyl-β-D-glucopyranoside. After incubation for 15 min on ice, lysates were centrifuged (12 000*g*, 4 °C for 10 min), and supernatants were collected and stored in SDS sample buffer in −20 °C. An aliquot of the supernatant was used to determine protein concentration by the Coomassie blue method.[36]

Protein samples (20 μg of lysates) were analyzed according to standard Western blotting protocol as described previously.[37] The membrane was incubated with the primary antibody in the blocking solution overnight at 4 °C and with the secondary antibody in the blocking solution for 1 h at room temperature. Bound antibody was detected using SuperSignal West Pico or Dura chemiluminescent substrate (Pierce, Rockford, U.S.A.) and Image Quant LAS 4000 mini imaging system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The quantitation of the chemiluminescent signal was carried out with the use of Imaging Quant TL software (GE Healthcare).

RNA Extraction and Quantitative RT-PCR. At the indicated time points, culture medium was removed, and total RNA extraction was carried out with GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, U.S.A.) according to the manufacturer's instructions. Total RNA was reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA). cDNA obtained from the RT-reaction was diluted 1:20 with RNase-free water and was subjected to quantitative PCR using TaqMan Universal PCR Master Mix and ABI PRISM 7000 Sequence detection system (Applied Biosystems, Foster City, CA, USA). Primers and probes (Table 2) for iNOS, IL-6, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, used as a control gene) were designed using Primer Express Software (Applied Biosystems, Foster City, CA, U.S.A.) and supplied by Metabion (Martinsried, Germany). Expression of MCP-1 mRNA was measured using TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA, U.S.A.).

The primer and probe sequences and concentrations were optimized according to manufacturer's guidelines in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C. PCR reaction parameters were as follows: incubation at 50 °C for 2 min, incubation at 95 °C for 10 min, and thereafter 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. Each sample was determined in duplicate. A standard curve method was used to determine the relative mRNA levels.

ELISA. IL-6 and MCP-1 were measured in the culture medium by enzyme-linked immunosorbent assay (ELISA) using reagents from R&D Systems Europe Ltd. (Abingdon, U.K.).

Carrageenan-Induced Paw Edema in Mice. Anti-inflammatory effects were studied by measuring carrageenan-induced paw edema in male C57BL/6NCrl mice (Scanbur, Copenhagen, Denmark). The study was carried out in accordance with the legislation for the protection of animals used for scientific purposes (directive 2010/63/EU) and The Finnish Act on Animal Experimentation (62/2006). The study was authorized in Finland by the National Animal Experiment Board, approval number ESAVI/5019/04.10.03/2012 (granted September third, 2012). Paw edema was induced under anesthesia, and all efforts were made to minimize suffering. Animals were housed under conditions of optimum light, temperature, and humidity (12:12 h light-dark cycle, 22 ± 1 °C, 50−60%) with food and water provided ad libitum. Eight-week old male mice were divided into three groups: control group, compound **9** (10 mg/kg) group, and dexamethasone (2 mg/kg) group. Mice were dosed with 500 μL of PBS-8% DMSO vehicle or the tested compound by intraperitoneal injection 2 h before carrageenan was applied. The mice were anesthesized by intraperitoneal injection of 0.5 mg/kg of medetomide (Domitor 1 mg/mL, Orion Oyj, Espoo) and 75 mg/kg of ketamine (Ketalar 10 mg/mL, Pfizer Oy Animal Health, Helsinki, Finland), and thereafter, the mice received 45 μL intradermal injection in one hindpaw of normal saline containing λ-carrageenan 1.5% (w/v). The contralateral paw received 45 μL of saline, and it was used as a control. Edema was measured before and 3 and 6 h after carrageenan injection by use of a plethysmometer (Ugo Basile, Comerio, Italy).

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Statistics. Results are expressed as the mean ± standard error of mean (SEM). Statistical significance of the results was calculated by one-way ANOVA with Dunnett's post test (dose curves) or Bonferroni's post test (multiple comparisons) by using GraphPad InStat 3 for Windows XP (Graph-Pad Software, San Diego, CA, U.S.A.). Differences were considered significant at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

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Author Contributions

⊥Equal contribution as the first author (M.L. and R.H.).

Author Contributions

‖Equal contribution as the last/senior author (J.Y.-K. and E.M.).

Notes

The authors declare no competing financial interest.

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PUBLICATION IV

IL-6 in Osteoarthritis: Effects of Pine Stilbenoids

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Article

IL-6 in Osteoarthritis: Effects of Pine Stilbenoids

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Abstract: Interleukin-6 (IL-6) is involved in the pathogenesis of various inflammatory diseases, like rheumatoid arthritis (RA). In the present study, we investigated the role of IL-6 in osteoarthritis (OA) patients and the effects of the stilbenoids monomethyl pinosylvin and pinosylvin on the expression of the cartilage matrix components aggrecan and collagen II and the inflammatory cytokine IL-6 in human OA chondrocytes. Synovial fluid and plasma samples were obtained from 100 patients with severe OA [BMI 29.7 (8.3) kg/m^2, age 72 (14) years, median (IQR); 62/38 females/males] undergoing total knee replacement surgery. IL-6 and matrix metalloproteinase (MMP) concentrations in synovial fluid and plasma were measured by immunoassay. The effects of pinosylvin on the expression of IL-6, aggrecan, and collagen II were studied in primary cultures of human OA chondrocytes. IL-6 levels in synovial fluid from OA patients [119.8 (193.5) pg/mL, median (IQR)] were significantly increased as compared to the plasma levels [3.1 (2.7) pg/mL, median (IQR)] and IL-6 levels in synovial fluid were associated with MMPs and radiographic disease severity. Natural stilbenoids monomethyl pinosylvin and pinosylvin increased aggrecan expression and suppressed IL-6 production in OA chondrocytes. The results propose that IL-6 is produced within OA joints and has an important role in the pathogenesis of OA. Stilbenoid compounds monomethyl pinosylvin and pinosylvin appeared to have disease-modifying potential in OA chondrocytes.

Keywords: interleukin-6; osteoarthritis; stilbenoids; pinosylvin; aggrecan; chondrocytes

1. Introduction

Interleukin-6 (IL-6) was cloned in 1980s and it was first shown to promote the activation of T and B lymphocytes as well as to regulate the inflammation-associated acute-phase response. Currently, IL-6 is known as a mediator of inflammation, immune response and hematopoiesis [1]. Targeting IL-6 has become important in the drug development because of the pathological role of IL-6 in numerous adverse conditions. Tocilizumab which is a humanized monoclonal antibody against IL-6 receptor, is used as a second-line treatment of rheumatoid arthritis (RA).

Osteoarthritis (OA) is the most common form of arthritis. The etiology of OA is still largely unknown although risk factors like certain genes, gender, age, joint trauma and obesity have been identified. Nowadays, there are no effective disease-modifying treatments except surgical interventions and the treatment is mainly limited to analgesics and other symptomatic approaches [2,3].

IL-6 is detected in synovial fluid and expressed in osteoarthritic cartilage which makes its inhibition an appealing potential target in the treatment of OA [4–7]. Recently it was published that inhibition of IL-6 by tocilizumab reduced pain behavior in a monosodium iodoacetate-induced

experimental model of OA in the rat, however, no clinical studies with IL-6 inhibitors in OA have been conducted to date [8]. Therefore, we were interested in studying the effects of the natural stilbenoids monomethyl pinosylvin and pinosylvin (Figure 1) in OA chondrocytes. Our hypothesis was supported by the fact that stilbenoids had been previously shown to inhibit the production of pro-inflammatory cytokines, including IL-6, in activated macrophages. Furthermore, resveratrol, the best known stilbenoid, structurally close to pinosylvin, has been shown to inhibit IL-6 in primary human chondrocytes [9–11].

In the present study, we report IL-6 concentrations in synovial fluid and plasma samples from 100 OA patients undergoing total knee replacement surgery and the association between the levels of IL-6, matrix metalloproteinases (MMPs) and the radiographic severity of the disease. In addition, we investigated the effects of monomethyl pinosylvin and pinosylvin on the expression of IL-6, aggrecan and collagen II in primary human OA chondrocytes.

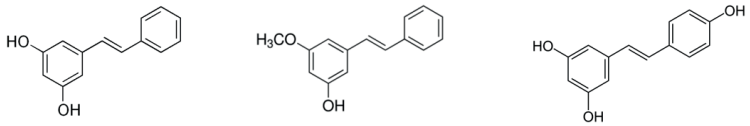


Figure 1. Chemical structures of pinosylvin, monomethyl pinosylvin and resveratrol.

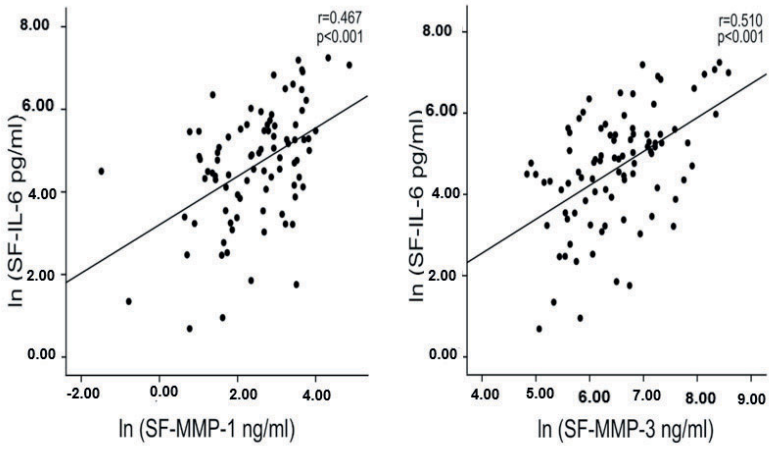
2. Results

2.1. IL-6 Concentrations in Synovial Fluid are Higher than those in Plasma in Patients with OA

In osteoarthritis patients (n = 100) undergoing knee replacement surgery, IL-6 concentrations in synovial fluid [119.8 (193.5) pg/mL, median (IQR)] were significantly higher than those in plasma [3.1 (2.7) pg/mL, median (IQR)]. No correlation between the synovial fluid and plasma levels were found suggesting that IL-6 is produced locally within the joint.

2.2. IL-6 Concentrations in Synovial Fluid Correlate with the Radiographic Severity of OA and with Matrix Metalloproteinase Concentrations

The preoperative knee radiographs were evaluated and Ahlbäck classification from grades 1 to 5 was used. Grades 1–3 and 4–5 were combined for the analysis. Mean synovial fluid IL-6 concentrations were higher (p = 0.027) in the group of grades 4 and 5 [234.1 (264.7) pg/mL, median (IQR)] than in the group of grade 1–3 [94.6 (183.0) pg/mL, median (IQR)] suggesting that IL-6 concentrations in synovial fluid are related to the disease severity. Furthermore, IL-6 in synovial fluid correlated with cartilage degrading matrix metalloproteinases MMP-1 (r = 0.467, $p < 0.001$) and MMP-3 (r = 0.510, $p < 0.001$) (Figure 2).



(a) **(b)**

Figure 2. Correlation between IL-6 and MMP-1 (**a**) and MMP-3 (**b**) in patients with osteoarthritis. IL-6 and MMP levels in synovial fluid (SF) were measured by immunoassay. Natural logarithms (LN) of the SF concentrations of IL-6 and MMPs were calculated in order to have normally distributed variables for the Pearson correlation analysis. In the Figure, correlation coefficients (r) and p values are given. Synovial fluid samples were collected from 100 patients with knee OA [BMI 29.7 (8.3) kg/m^2, age 72 (14) years, median (IQR); 62/38 females/males].

2.3. Monomethyl Pinosylvin, Pinosylvin and Resveratrol Suppress IL-6 Expression in Primary Cultures of OA Chondrocytes

Primary chondrocytes from OA patients produced IL-6 and that was significantly increased when the cells were exposed to the pro-inflammatory cytokine IL-1β or IL-17, both involved in the pathogenesis of OA [12]. Next, the effect of pine stilbenoids monomethyl pinosylvin and pinosylvin which are structurally close to the better known stilbenoid compound resveratrol were studied in cultures of primary human OA chondrocytes. Monomethyl pinosylvin and pinosylvin inhibited IL-6 expression at mRNA (Figure 3a,b) and protein level (Figure 3c,d) in both IL-1β and IL-17 stimulated chondrocytes as did the control stilbenoid resveratrol. Dexamethasone as a standard anti-inflammatory compound had an anticipated inhibitory effect also.

2.4. Pine Stilbenoids Inhibit NF-κB Mediated Transcription in Human Chondrocytes

NF-κB is a key transcription factor regulating IL-6 production [1]. Therefore we investigated the effects of monomethyl pinosylvin and pinosylvin on NF-κB mediated transcription. T/C28a2 human chondrocyte cell line was engineered to express luciferase (LUC) gene under the control of an NF-κB driven promoter. Monomethyl pinosylvin and pinosylvin significantly inhibited NF-κB mediated transcription (measured as luciferase activity) (Figure 4). The inhibitory effect was similar with ammonium pyrrolidine dithiocarbamate (PDTC), a known NF-κB inhibitor.

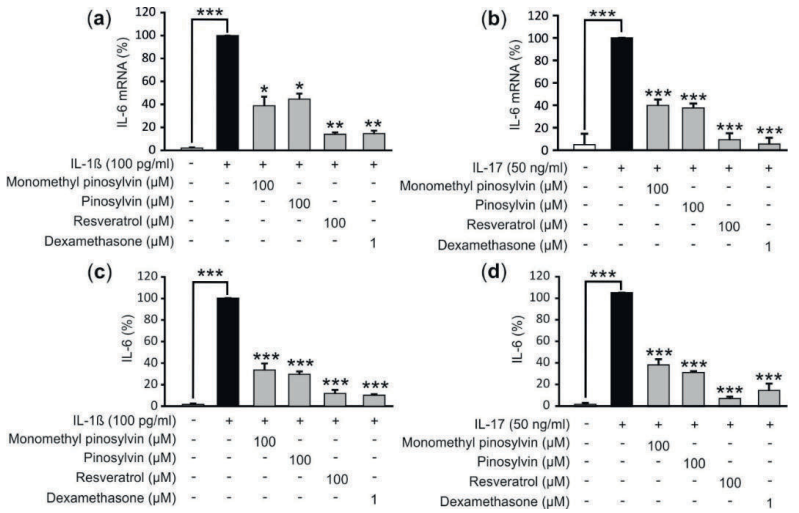


Figure 3. Effects of monomethyl pinosylvin, pinosylvin, resveratrol and the anti-inflammatory control compound dexamethasone in IL-1β and IL-17 stimulated primary human OA chondrocytes on IL-6 expression at mRNA (**a**,**b**) and protein (**c**,**d**) level at time point 24 h. IL-6 mRNA was measured by quantitative reversed transcriptase polymerase chain reaction (RT-PCR) and the results were normalized against GAPDH mRNA. IL-6 concentrations in the culture media were measured by immunoassay. IL-6 levels were 12.5–25.8 ng/mL in IL-1β and 2.6–10.2 ng/mL in IL-17 stimulated cells in the absence of the tested compounds. Primary chondrocytes were isolated from cartilage samples obtained from three consequent donors and the experiments were performed in duplicate; $n = 3$ was used in the calculations. Results are expressed as mean +SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ as compared to cells treated with IL-1β or IL-17 only.

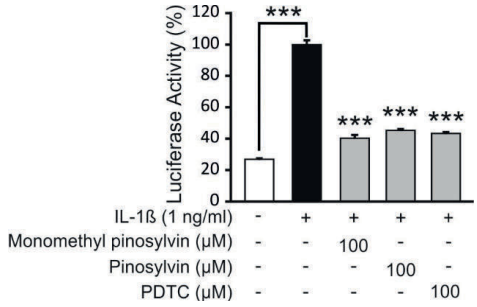


Figure 4. Effects of monomethyl pinosylvin and pinosylvin on NF-*k*B-mediated transcription in human T/C28a2 chondrocytes transfected with luciferase reporter construct. T/C28a2pGL4.32NFκB cells were stimulated with IL-1β in the presence of the pine stilbenoids or the known NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) for 5 h and luciferase activity was measured. Results are presented as mean +SEM, $n = 4$, *** $p < 0.001$ as compared to cells incubated with IL-1β only. The inhibitory effect was similar with ammonium pyrrolidine dithiocarbamate (PDTC), a known NF-κB inhibitor.

2.5. Pinosylvin, Monomethyl Pinosylvin and Resveratrol Enhance the Expression of the Anabolic Factor Aggrecan in Human Primary Chondrocytes

Aggrecan and collagen II are both major components of extracellular matrix in the cartilage [13]. IL-1β and IL-17 decreased the synthesis of the two anabolic factors in OA chondrocytes (Figure 5), as expected [12]. Interestingly, monomethyl pinosylvin, pinosylvin and resveratrol increased the

aggrecan expression in non-stimulated cells and reversed the suppressive effect of IL-1β and IL-17 on aggrecan expression in OA chondrocytes but had no effect on collagen II expression (Figure 5). The control compound dexamethasone also enhanced aggrecan but not collagen expression.

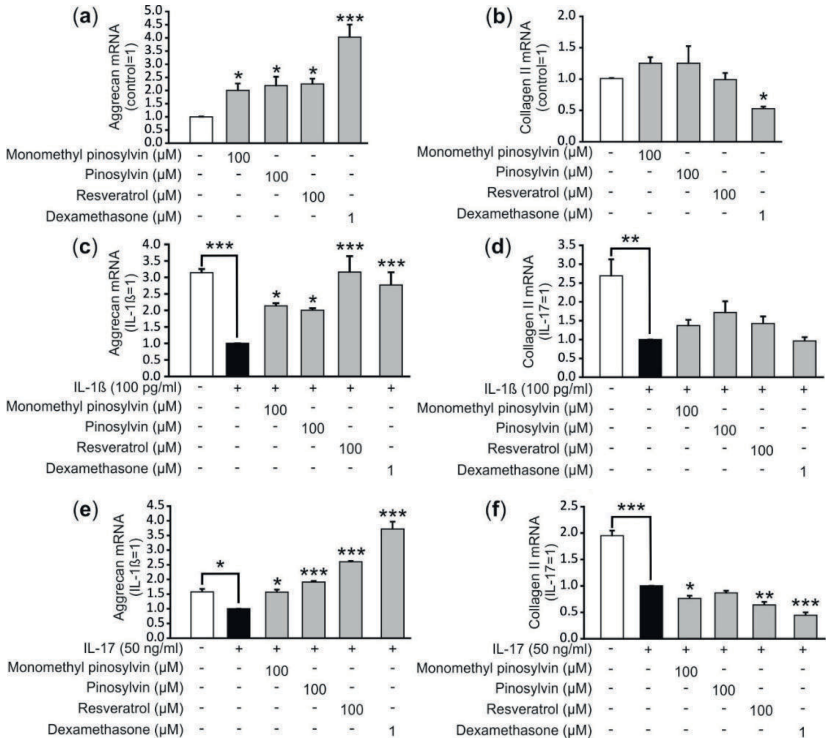


Figure 5. Effects of monomethyl pinosylvin, pinosylvin, resveratrol and the anti-inflammatory control compound dexamethasone on aggrecan and collagen II expression. Human primary chondrocytes were cultured with the tested compounds alone (**a**,**b**) or with IL-1β (**c**,**d**) or IL-17 (**e**,**f**) for 24 h before RNA was extracted. Aggrecan and collagen II mRNA was determined by reversed transcriptase polymerase chain reaction (RT-PCR) and the results were normalized against GAPDH mRNA. Primary chondrocytes were isolated from cartilage samples obtained from three consequent donors and the experiments were performed in duplicate; $n = 3$ was used in the calculations. Results are expressed as mean +SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ as compared to cells non-stimulated or treated with IL-1β or IL-17 only.

3. Discussion

In OA patients, IL-6 levels in synovial fluid were significantly higher than those in plasma and correlated positively with MMP enzymes (Figure 2) and radiographic severity of OA; while plasma IL-6 concentrations in OA patients were comparable to those reported in healthy individuals [2]. The importance of IL-6 in OA is also supported by previous results. It was shown that IL-6 concentrations in synovial fluid were considerably higher in patients with cartilage defect or OA than in donors without joint pathology [14,15]. Follow-up study showed that increased serum concentrations of IL-6 were associated with articular changes observed in radiographs [16]. In our study, advanced radiographic severity of OA was associated with higher IL-6 concentrations in synovial fluid but not in plasma. Clinical observations together with the present findings strongly support the important role of IL-6 in the pathogenesis of and as a potential drug target in OA.

Stilbenoids are naturally occurring compounds found in grapes, almond, rhubarb and berries which makes them part of our normal diet. Stilbenoids are also secondary products of heartwood formation in trees where they act as phytoalexins. Two silbenoids monomethyl pinosylvin and pinosylvin isolated from the knots of Scots pine (*Pinus sylvestris*) were identified to have anti-inflammatory potential in our previous studies [9]. Therefore we aimed to study their effects on chondrocytes, cell type significantly involved in the pathogenesis of OA.

To our knowledge, the effects of monomethyl pinosylvin and pinosylvin have not previously been studied in OA. Whereas another stilbenoid, resveratrol, has been studied in arthritis models. In a surgically induced OA model in mice, resveratrol decreased destruction of articular cartilage, production of the catabolic factor MMP-13 and expression of the inflammatory enzyme iNOS [17]. Furthermore resveratrol increased thickness of the calcified cartilage and improved Mankin scores [17]. In another study, Mankin score improval and inhibition of cartilage destruction was seen in a surgical model of OA in rabbits after intra-articular resveratrol treatment [18]. Interestingly, resveratrol was found effective also in the prevention of collagen-induced arthritis model in mice [19]. Incidence and severity of arthritis as well as the amount of infiltrated cells in the joint were decreased after 8 weeks treatment with resveratrol and, cartilage and bone erosions and synovial hyperplasia were prevented [19].

In the present study, the pine stilbenoids monomethyl pinosylvin and pinosylvin, as well as resveratrol, were found to suppress IL-6 expression in primary OA chondrocytes stimulated with IL-1β or IL-17 (Figure 3). There is a very limited amount of data available of other stilbenoids than resveratrol in chondrocytes but resveratrol has been shown to reduce MMPs and IL-6 in human chondrocyte cultures supporting our finding [11,20]. Monomethyl pinosylvin and pinosylvin suppressed IL-6 expression possibly via a mechanism involving the inhibition of NF-κB activity (Figure 4). Both pine stilbenoids inhibited NF-κB mediated transcription in human chondrocyte cell line and NF-κB is a known transcription factor regulating IL-6 production [1].

The physical function of joints and biochemical properties of cartilage are critically reliant on the integrity of the extra cellular matrix (ECM). In normal conditions, articular chondrocytes preserve a dynamic balance between degradation and synthesis of ECM components. ECM is composed of a collagenous network, mostly type II collagen, alongside with glycosaminoglycans like hyaluronan, and a variety of proteoglycans including aggrecan. In OA, on the other hand, the equilibrium has been disrupted and catabolic processes are accelerated while anabolic processes are suppressed. [21]

Aggrecan mRNA expression was upregulated by monomethyl pinosylvin, pinosylvin and resveratrol in unstimulated and IL-1β or IL-17 stimulated primary human chondrocytes (Figure 5). The effect is beneficial to the cartilage homeostasis. Aggrecan as a component of proteoglycans is essential to maintain the normal function of articular cartilage because it draws water into cartilage matrix and forms a hydrated gel structure that provides the cartilage with load-bearing properties [21]. The increased aggrecan expression might also be a positive consequence of IL-6 inhibition because IL-6 has been shown to suppress proteoglycan production in murine bone marrow-derived mesenchymal stem cells [22]. There is also a previous study where resveratrol increased aggrecan expression similar to in our study but contrary to our study it also increased the collagen II expression [16]. One possible explanation for the differing result might be that they used non-arthritic articular cartilage to isolate primary chondrocytes while we had OA cartilage.

It has been proposed that OA is complicated with IL-6 induced oxidative stress. IL-6 together with IL-1 dysregulates the antioxidant defense mechanisms in chondrocytes and increases the production of reactive oxygen species (ROS) [23,24]. ROS mediate intracellular events and regulate gene expression including MMPs supporting the degradation of cartilage matrix [25,26]. Free radicals can also attack directly proteoglycan and collagen molecules in ECM [26]. Thereby inhibition of mechanisms (such as IL-6) able to trigger ROS production and suppress antioxidant defense could be a reasonable target to prevent or treat OA.

Our results suggest that monomethyl pinosylvin and pinosylvin may have disease-modifying properties in OA chondrocytes through down-regulation of IL-6 and up-regulation of aggrecan. Interestingly, it has been recently reported that monomethyl pinosylvin inhibits TRPV1 activator capsaicin induced pain behavior [27] and that pinosylvin suppresses TRPA1-mediated ion currents in vitro and TRPA1-mediated acute paw inflammation in mice [28]. Therefore, it is tempting to speculate that in addition of being possible disease-modifying OA drug candidate stilbenoids might have also pain relieving properties in OA.

In conclusion, the present findings indicate that IL-6 is produced within OA joints, and it is associated with increased levels of cartilage degrading MMP enzymes and with the severity of radiographically detected joint changes in patients with OA. For the first time monomethyl pinosylvin and pinosylvin were shown to inhibit IL-6 production and increase aggrecan expression in primary human OA chondrocytes. The results suggest an important role for IL-6 in the pathogenesis of OA and the potential of pine stilbenoids as disease-modifying compounds in OA chondrocytes.

4. Materials and Methods

4.1. Chemicals

Pinosylvin and monomethyl pinosylvin were obtained from Arbonova (Turku, Finland) and resveratrol from Tocris Bioscience (Ellisville, MS, USA). All other reagents were from Sigma Chemical Co (St. Louis, MO, USA) unless otherwise stated.

4.2. Patients and Clinical Samples

The patients fulfilled the American College of Rheumatology classification for OA [29]. Blood and synovial fluid samples were obtained from 100 OA patients [BMI 29.7 (8.3) kg/m^2, age 72 (14) years, median (IQR); 62/38 females/males] undergoing total knee replacement surgery. Plasma and synovial fluid samples were stored at −80 °C until analyzed. The study was approved by the Ethics Committee of Tampere University Hospital, Tampere, Finland (ethics code R06223), and was conducted in accordance with the Declaration of Helsinki. All patients provided their written informed consent.

4.3. Primary Chondrocyte Experiments

Primary chondrocyte experiments were performed as previously described by Koskinen et al. [30]. Briefly, leftover pieces of OA cartilage from knee joint replacement surgery were used under full patient consent and approval by the Ethics Committee of Tampere University Hospital, Tampere, Finland (ethics code R09116). Full-thickness pieces of articular cartilage from femoral condyles and tibial plateaus showing macroscopic features of early OA were removed aseptically from subchondral bone with a scalpel, and cut into small pieces. Cartilage pieces were washed with PBS and chondrocytes were isolated by enzymatic digestion for 16 h at 37 °C in a shaker by using a collagenase enzyme blend (1 mg/mL Liberase Research Grade medium; Roche, Mannheim, Germany). Isolated chondrocytes were washed and plated on 24-well plates (1.5 × 105 cells/mL) in culture medium (DMEM with glutamax-I Gibco supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), and amphotericin B (250 ng/mL) (all from Thermo Fisher Scientific, Carlsbad, CA, USA) containing 10% fetal bovine serum (Lonza, Verviers Sprl, Verviers, Belgium). Samples for each primary chondrocyte experiment were obtained from three consequent donors and the cell culture experiments were performed in duplicate; $n = 3$ was used in the calculations. All measured values were included for the final results. Chondrocytes were stimulated with IL-1β (100 pg/mL) or IL-17 (50 ng/mL) both from R&D Systems Europe Ltd. (Abingdon, UK) with and without the tested compounds for 24 h. The culture media were stored at −20 °C until analyzed. Cytotoxicity of the investigated compounds was ruled out by measuring cell viability using Cell Proliferation Kit II (Roche) according to the manufacturer's instructions.

4.4. Preparation of the Stable T/C28a2pGL4.32NFκB Cell Line

In order to investigate the effects of the stilbenoids on the NF-κB mediated transcription, the T/C28a2 human chondrocyte cell line kind gift from Professor Mary B. Goldring were stably transfected with luciferase reporter construct, pGL4.32[luc2P/NF-κB-RE/Hygro] [31]. The plasmid was purchased from Promega Corporation (Madison, WI, USA) and contains five copies of an NF-κB response element that drives transcription of the luciferase reporter gene. T/C28a2 human chondrocyte cell line was cultured at 37 °C in 5% CO_2 atmosphere and grown in DMEM/Ham's F12 (1:1) containing 5% heat-inactivated foetal bovine serum, penicillin (100 units/mL), streptomycin (100 μg/mL) and amphotericin B (250 ng/mL). Cells were seeded on 24 well plates and cell monolayers were grown for 72 h to confluence before the experiments were started and the compounds of interest were added in fresh culture medium. Firefly luciferase activity was measured using the luciferase assay reagent (Promega Corp., Madison, WI, USA), and the results were normalized to the total cellular protein.

4.5. Measurement of IL-6 and MMPs by Immunoassay

Concentration of IL-6 in plasma, synovial fluid, and culture media was measured by enzyme-linked immunosorbent assay (ELISA) with commercial reagents from Sanquin (Amsterdam, The Netherlands). The detection limit for IL-6 was 0.3 pg/mL. MMP-1 concentrations in the synovial fluid were determined by Multiplex bead array (Fluorokine® Human MMP Multi Analyte Profiling Base Kit, R&D Systems, Inc., Minneapolis, MN, USA) and MMP-3 concentrations were assessed by ELISA (R&D Systems, Inc.). Detection limits were 10.7 pg/mL for MMP-1 and 15.6 pg/mL for MMP-3.

4.6. Measurement of IL-6, Collagen II and Aggrecan mRNA Levels

At the indicated time points, culture medium was removed from primary human OA chondrocytes and total RNA was extracted with GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions. Total RNA was reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA, USA). cDNA obtained from the RT-reaction was diluted 1:20 with RNAse-free water and subjected to quantitative PCR using TaqMan Universal PCR Master Mix and ABI PRISM 7000 Sequence detection system (Applied Biosystems). Primers and probes (Table 1) for IL-6, aggrecan, collagen II and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, used as a control gene) were designed using Primer Express® Software (Applied Biosystems) and supplied by Metabion (Martinsried, Germany).

Table 1. Primer and probe sequences.

Gene	Oligonucleotide	Sequence 5′→3′
Human Interleukin-6	Forward primer	TACCCCCAGGAGAAGATTCCA
	Reverse primer	CCGTCGAGGATGTACCGAATT
	Probe	CGCCCCACACAGACAGCCACTC
Human Collagen II	Forward primer	GGCAATAGCAGGTTCACGTACA
	Reverse primer	CGATAACAGTCTTGCCCCACTT
	Probe	CTGAAGGATGGCTGCACGAAACATACC
Human Aggrecanase	Forward primer	GCCTGCGCTCCAATGACT
	Reverse primer	TAATGGAACACGATGCCTTTCA
	Probe	CCATGCATCACCTCGCAGCGGTA
Human GAPDH *	Forward primer	AAGGTCGGAGTCAACGGATTT
	Reverse primer	GCAACAATATCCACTTTACCAGAGTTAA
	Probe	CGCCTGGTCACCAGGGCTGC

* GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

The primer and probe sequences and concentrations were optimized according to manufacturer's guidelines in TaqMan Universal PCR Master Mix Protocol part number 4,304,449 revision C. PCR reaction parameters were as follows: incubation at 50 °C for 2 min, incubation at 95 °C for 10 min, and thereafter 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. A standard curve method was used to determine the relative mRNA levels.

4.7. Statistical Analysis

SPSS program version 17.0 for Windows software (SPSS Inc, Chicago, IL, USA) was used for analyzing clinical data. Normality of the data was tested by Kolmogorov-Smirnov test. Based on that, nonparametric tests were used in the analysis. Differences between groups were tested by Wilcoxon Signed Rank Test. Pearson's r was used to analyse correlation after natural logarithm (LN) transformation by which normal distribution was achieved. P-values less than 0.05 were considered significant.

Chondrocyte results are expressed as the mean ± standard error of mean (SEM). Statistical significance of the results was calculated by one-way ANOVA with Bonferroni's post-test by using GraphPad InStat 3 for Windows XP (Graph-Pad Software, San Diego, CA, USA). Differences were considered significant at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

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