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In Vitro Immunopharmacological Profiling of Ginger (*Zingiber officinale* Roscoe)

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Summary

In traditional medicine the rhizome of ginger (*Zingiber officinale* Roscoe) has been used for centuries against inflammatory diseases. To date, not much is known about the underlying molecular mechanisms for the therapeutic effects attributed to ginger. This work shows by the use of an *in vitro* model that the dominant constituents of the ginger rhizome are likely to be absorbed, but show high affinity for the intestinal mucosa. Ginger extracts and isolated compounds also exert a marked inhibition of cytokine induction in human whole blood. Especially the inhibition of IL-1β (≥ 35 %) is nearly independent of the used stimuli and could be correlated to the inhibition of intracellular phospholipases A_2 (PLA_2) by means of a mixed micelle assay specific for certain PLA_2 groups. i- and cPLA_2 inhibition by ginger extract (10 µg/ml) and selected isolated constituents (10 µM) was approximately 50%. Furthermore, the effects of secondary metabolites of the ginger rhizome on serotonin 5-HT_{1A} receptors (5-HT_{1A}R) were examined. It is shown that ginger extracts and isolated phenylpropanoids bind in the low µM range to 5-HT_{1A}R and that 10-gingerol, 10-shogaol, 1-dehydro-6-gingerdiol, and ginger extracts partially activate 5-HT_{1A}R *in vitro*. Additionally, calcium-mediated T cell activation, MAP kinase phosphorylation, lymphocyte proliferation, IL-1β transcription and translation, and ATP-mediated ion fluxes were examined, but showed no significant modulation by ginger extracts and isolated constituents. In relation to common *in vitro* studies of botanical drugs, the molecular mechanisms of arabinogalactan-proteins (AGPs) as immunostimulatory agents were studied. It was shown *in vitro* and *in vivo* that sugar moieties on common cell wall AGPs activate innate immune responses (e.g. monokine expression, NO production, and edema formation). These processes are Toll-like receptor 4 mediated and can be reproduced in whole blood, isolated monocytes, and in mice. Finally, these AGPs are not absorbed and their pro-inflammatory *in vitro* effects are a conserved phenomenon found with all plant AGPs.

Zusammenfassung

Das Ingwerrhizom (*Zingiber officinale* Roscoe) wird in der Volksmedizin seit Jahrhunderten gegen entzündliche Erkrankungen eingesetzt. Über die molekularen Mechanismen der dem Ingwer zugeschriebenen therapeutischen Effekte ist bis anhin jedoch nicht viel bekannt. In dieser Arbeit wurde anhand eines *in vitro* Models gezeigt, dass die wichtigsten Inhaltsstoffe des Ingwerrhizoms wahrscheinlich resorbiert werden, jedoch eine hohe Affinität zur intestinalen Mukosa aufweisen. Ingwerextrakte und isolierte Inhaltsstoffe zeigen eine merkliche Hemmung der induzierten Cytokinproduktion in humanem Vollblut. Hier sticht die Inhibition von IL-1β (≥ 35 %) hervor, welche fast unabhängig vom verwendeten Stimulus ist. Dies konnte, anhand eines Gruppen-spezifischen Tests mit gemischten Mizellen, mit der Inhibition von intrazellulären Phospholipasen A_2 (PLA_2) korreliert werden. Die Hemmung der i- und $cPLA_2$ durch einen Ingwerextrakt (10 μg/ml) und durch ausgewählte isolierte Inhaltsstoffe (10 μM) war annähernd 50%. Des Weitern wurden Effekte von Sekundärmetaboliten aus dem Ingwer-Rhizom an Serotonin 5-HT_{1A} Rezeptoren (5-HT_{1A}R) untersucht. Es wurde gezeigt, dass Ingwerextrakte und isolierte Phenylpropanoide im tiefen μM Bereich an den 5-HT_{1A}R binden und dass der 5-HT_{1A}R *in vitro* von 10-Gingerol, 10-Shogaol, 1-Dehydro-6-gingerdiol und einem Ingwerextrakte partiell aktiviert wird. Ausserdem wurden die calciumvermittelte T-Zell Aktivierung, die MAP Kinasen Phosphorylierung, die Lymphocyten-Proliferation, die IL-1β Transkription und Translation sowie ATP-vermittelte Ionenflüsse untersucht. Diese zeigten jedoch keine signifikante Modulation durch Ingwerextrakte und isolierte Inhaltsstoffe. Des Weiteren wurde, im Zusammenhang mit gängigen *in vitro* Untersuchungen von pflanzlichen Drogen, der molekulare Mechanismus von Arabinogalactan-Proteinen (AGPs) als Immunstimulanzien untersucht. Es wurde sowohl *in vitro* als auch *in vivo* gezeigt, dass Zuckerreste an gängigen Zellwand-gebundenen AGPs die angeborene Immunantwort (z.B. Monokin-Expression, NO Produktion und Ödembildung) aktivieren. Diese Prozesse laufen über den Toll-like Rezeptor 4 und lassen sich sowohl in Vollblut, isolierten Monozyten als auch in Mäusen reproduzieren. Zusammenfassend lässt sich sagen dass AGPs nicht resorbiert werden und ihre pro-inflammatorischen *in vitro* Effekte ein phylogenetisch unverändertes Phänomen sind, welches durch alle pflanzlichen AGPs verursacht wird.

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Last but not least, I thank all those who supported me in any respect and contributed to the completion of this work.

List of Abbreviations

5-HETE: 5-Hydroxy eicosatetraenic acid

5-HT: Serotonin (5-hydroxytryptamine)

5-HT_XR: Serotonin receptor (e.g. 5-HT_{1A}R: serotonin receptor subtype 1A)

6-DHP: 6-Dihydroparadol (α-heptyl-4-hydroxy-3-methoxy-benzenepropanol)

6-G: 6-Gingerol (5-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-decan-3-one)

6-GD: 6-Gingerdiol (1-(4-hydroxy-3-methoxyphenyl)-decan-3,5-diol)

6-GDO: 1-Dehydro-6-gingerdione (1-(4-hydroxy-3-methoxyphenyl)-1-decene-3,5-dione)

6-P: 6-Paradol (1-(4-hydroxy-3-methoxyphenyl)-decan-3-one)

6-S: 6-Shogaol (1-(4-hydroxy-3-methoxyphenyl)-4-decen-3-one)

8-DHP: 8-Dihydroparadol (α-nonyl-4-hydroxy-3-methoxy-benzenepropanol)

8-G: 8-Gingerol (5-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-dodecan-3-one)

8-GDO: 1-Dehydro-8-gingerdione (1-(4-hydroxy-3-methoxyphenyl)-1-dodecene-3,5-dione)

8-S: 8-Shogaol (1-(4-hydroxy-3-methoxyphenyl)-4-dodecen-3-one)

10-DHP: 10-Dihydroparadol (α-undecyl-4-hydroxy-3-methoxy-benzenepropanol)

10-G: 5-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-tetradecan-3-one (10-gingerol)

10-GDO: 1-Dehydro-10-gingerdione (1-(4-hydroxy-3-methoxyphenyl)-1-tetradecene-3,5-dione)

10-S: 10-Shogaol (1-(4-hydroxy-3-methoxyphenyl)-4-tetradecen-3-one)

α-CD28: anti-CD28 antibody

α-CD3: anti-CD3 antibody

AA: Arachidonic acid

AcOH: Acetic acid

ADP: Adenosine 5'-O-diphosphate

AGP: Arabinogalactan-protein

APC: Antigen presenting cells

ATP: Adenosine 5'-O-triphosphate

ATRA: all-*trans*-Retinoic acid

AUC: Area under the curve

BCR: B cell receptor

BSA: Bovine serum albumin

$[Ca^{2+}]_i$: Free intracellular calcium concentration

cADPR: Cyclic adenosine 5'-diphosphate ribose

cAMP: 3'-5'-Cyclic adenosine monophosphate

CD: Cluster of differentiation

CICR: Calcium-induced calcium release

CNS: Central nervous system

COX: Cyclooxygenase

$cPLA_2$: Cytosolic phospholipase A_2

CRAC channels: Calcium-release activated calcium channels

DAG: Diacyl glycerol

DAMP: Danger-associated molecular pattern

DBA: 2,4'-Dibromoacetophenone

DC: Dendritic cells

DCM: Dichloromethane

DMSO: Dimethylsulfoxide

DPAT: 8-Hydroxy-2-(dipropylamino)tetralin hydrobromide

DSPA: Distearyl phosphatidic acid (1,2-distearoyl-*sn*-glycero-3-phosphatidic acid)

DTNB: 5,5'-Dithio-bis(2-nitrobenzoic acid) or Ellmann's reagent

DTP: 2,2'-Dithiopyridine

DTT: 1,4-Dithio-DL-threitol

EDTA: Ethylenediaminetetraacetic acid

EGTA: Ethylene glycol tetraacetic acid

ERK: Extracellular signal-regulated kinases

Et_2O: Diethyl ether

EtOAc: Ethyl acetate

EtOH: Ethanol

FCS: Fetal calf serum

FFA: Free fatty acids

fMLP: N-Formylmethionine leucyl-phenylalanine

GDP: Guanosine 5'-O-diphosphate

GM-CSF: Granulocyte-macrophage colony-stimulating factor

GPCR: G protein coupled receptor

GTP: Guanosine 5'-O-triphosphate

GTPγS: Guanosine 5'-O-[gamma-thio]triphosphate

HEPES: 2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid

HMG: High-mobility group

HPLC: High-performance liquid chromatography

ICE: Interleukin 1 cleaving enzyme

IFN: Interferon

IL: Interleukin

iNOS: Inducible nitric oxide synthetase

IP3: Inositol 1,4,5-triphosphate

IP_3R: Inositol 1,4,5-triphosphate receptor

$iPLA_2$: Calcium-independent phospholipase A_2

JNK: c-Jun N-terminal kinase

LOX: Lipoxygenase

LPS: Lipopolysaccharide

LTB_4: Leukotriene B_4

Lyso-PC: Lysophosphatidylcholine

MΦ: Macrophages

MHC I and II: Major histocompatibility complex 1 and 2

MAFP: Methoxy arachidonyl fluorophosphonate

MAP2K, MAP3K, and MAP4K: MAPK kinases

MAPK: Mitogen-activated protein kinase

MeOH: Methanol

MOPS: 3-(N-Morpholino)-propanesulfonic acid

MtBE: Methyl t-butyl ether

NAADP: Nicotinic acid adenine dinucleotide phosphate

NF-κB: Nuclear factor κB

NK cells: Natural killer cells

NKT cells: Natural killer-like T cells

NMR: Nuclear magnetic resonance

NO: Nitric oxide

NSAID: Non-steroidal anti-inflammatory drug

p38: p38 Kinase (MAPK)

PAF: Platelet activating factor

PAF-AH: PAF-acetyl hydrolase (PLA_2 groups VII and VIII)

PAK: Palmitatoyl-6-*O*-ascorbate potassium salt

PAMP: Pathogen-associated molecular pattern

PBS: Phosphate-buffered saline

PC: Phosphatidylcholine

PC-PLC: Phosphatidylcholine specific phospholipase C

PEA: Palmitoylethanolamide

PG: Prostaglandin

PHA: Lectin from *Phaseolus vulgaris* also known as phytohaemagglutinin

PI3K: Phosphoinositide 3-kinase

PKC: Protein kinase C

PL: Phospholipase

PLA: Phospholipase A

PLC: Phospholipase C

PMA: Phorbol 12-myristate 13-acetate (12-O-tetradecanoylphorbol-13-acetate, TPA)

PTK: Protein tyrosine kinase

ROS: Reactive oxygen species

PRR: Pathogen recognition receptor

Rh123: Rhodamine 123

RyR: Ryanodine receptor

SDS: Sodium lauryl sulfate

SERCA: Sarcoplasmatic/endoplasmatic reticulum calcium ATPase

SOC: Store-operated channels

$sPLA_2$: Secreted phospholipase A_2

TACE: TNF-α cleaving enzyme

Tc: Cytotoxic T cells

T_H cells: T helper cells

TCM: Traditional Chinese medicine

TCR: T cell receptor

THF: Tetrahydrofurane

Thio-PC: 1-Hexadecyl-2-arachidonolythio-2-deoxy-*sn*-glycero-3-phosphorylcholine

TLC: Thin layer chromatography

TLR: Toll-like receptor

TNF: Tumor necrosis factor

T_{reg}: Regulatory T cells

TRIS: Tris(hydroxymethyl)-aminomethane

TRP: Transient receptor potential

TX: Thromboxane

1. Introduction

1.1 Ginger (*Zingiber officinale*)

Zingiber officinale (Roscoe) is a monocotyledon plant that belongs to the family of the *Zingiberaceae,* which consists of more than 1200 plant species in 53 genera. They include also other medical plants and well known spices like turmeric (*Curcuma longa*), lesser galangal (*Alpinia officinarum*), or cardamom (*Elettaria cardamomum* var. *minuscula*). The genus *Zingiber* was named after the Sanskrit word *zindschi* (hornshaped) by the English botanist William Roscoe (1753-1831) in a report published in 1807[1].

Ginger is propagated by scions and cultivated in many different cultivars, which vary strongly in the composition of their essential oil, water content, size, and the amount of oleoresin, which is composed of the non-volatile lipophilic constituents. With regard to the medical use, the term ginger usually denotes only the rhizome (Fig. 1 A) of the cultivars 'garden ginger' *Zingiber officinale* (syn. *Amomum angustifolium* Salisb., *Amomum zingiber* L., *Zingiber blancoi* Massk.) (Fig. 1 B), which are the most frequently used ginger species worldwide.

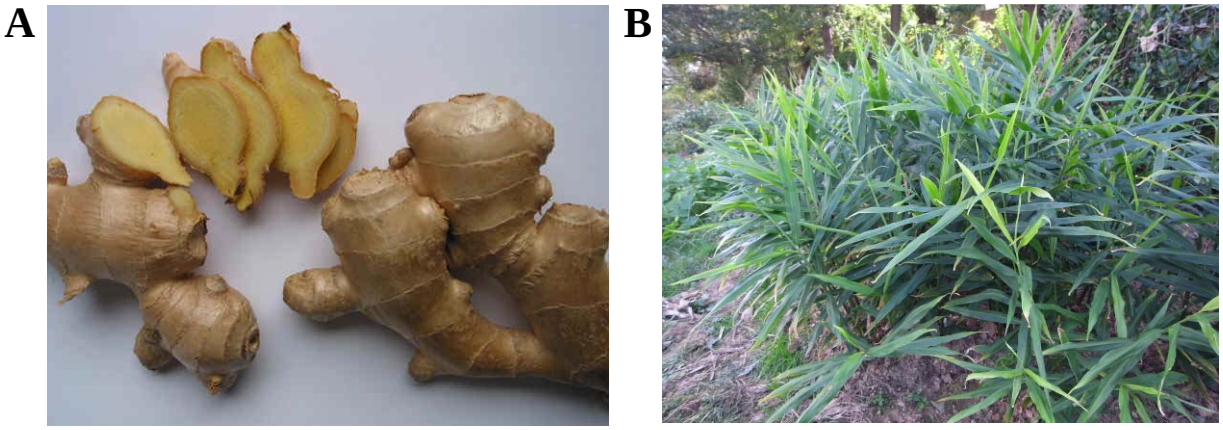


Figure 1: *Zingiber officinale*

A Fresh ginger rhizome from China. The slices are around 2 – 3 cm in diameter. **B** The aerial part of garden ginger, a herbaceous perennial plant with a reed-like appearance. © by jardintropical.over-blog.com

1.1.1 Origin

Ginger probably originates from South-East Asia. The ancient Greeks and Romans brought the rhizome to Southern Europe. Already in the 11th century it is mentioned in Anglo-Saxon veterinary pharmacopoeias and leech books, in the 13th century it was well known in all of Europe, and the Spanish established first plantations in the West Indies (mainly Jamaica) and in Mexico in the 16th century. Nowadays ginger is cultivated in the tropical parts of the world, from Asia to Africa, and large parts of South and Central America; mainly in India, in southern China, Indonesia, Nepal, and Nigeria. The best quality is said to come from Jamaica. [2, 3]

1.1.2 Description

Ginger is a perennial, erect growing reed-like plant with a thick subterranean, sympodial, in one plane branched rhizome with a thin dirty grey, yellow, pale silver to gold brown, or brown bark. The annual stems grow up to 1.5 m in height and have linear lanceolate sheathing leaves (5 to 30 cm long and 8 to 20 mm wide) which are alternatingly arranged, with a smooth surface, straight borders and of a pale green colour.

The rarely occurring flower stems are around 30 cm high. They bear a few bright yellow flowers with red labia being arranged in a small cone of obtuse bracts at its end (Fig. 2) [2, 4]. The odour of ginger is characteristically aromatic and spicy and varies depending on the origin and cultivar from lemonlike to tart or earthy. Its taste is pungent and aromatic.

The ginger rhizome is traded in several commercial grades [5] as the whole rhizome, with laterally or totally removed cork, in slices, as dried powder, or as candied cooked pieces.

Figure 2: The flowering plant of *Z. officinale* from a reprint of a coloured lithography in [2].
1 flower bud; 2 flower; 3 outer perigone, spread; 4 longitudinal section of flower with the small dentiform and sterile outer and the fertile inner stamens; 5 honey lip with small dentiform and sterile outer stamens; 6 pistil with small sterile stamens standing on the ovary; 7 upper part of the pistil with the stigma; 8 and 9 pistil in longitudinal and transverse section.
Adapted from an original painting of Prof. Schmidt in Berlin; coloured print of Mr. E. Günther in Gera.

1.1.3 Chemical Constituents

The ginger rhizome contains 0.6 to 3.3 % essential oil, comprising more than 150 secondary metabolites. This includes bisabolane-type sesquiterpenes (Fig. 3 A) like α-zingiberene (about 30 %), β-sesquiphellandrene (15-20 %), β-bisabolene (10-15 %), and ar-curcumene, bicyclic sesquiterpenes like zingiberol (a mixture of β-eudesmol with *trans* and *cis* ring juncture) as main odoriphore, and monoterpenes (Fig. 3 B) like citral (~20 % neral and 30-40 % geranial), which is responsible for the 'lemony' aroma of ginger, D-phellandrene as another important odoriphore, geraniol, borneol, α-terpineol, 1,8-cineol and its esters, and D-camphene.

The medically important and pungent tasting constituents of the ginger rhizome are part of the so called oleoresin, an oil which is not water vapour volatile.

The oleoresin is present at 5 to 8 % in the dry rhizome and contains a broad spectrum of phenylpropanoids. Around one quarter is 6-gingerol (6-G, Fig. 3 C a), besides homologues with longer alkyl chains and anhydro-derivatives (shogaols, which form mainly upon storage, Fig. 3 C b) and several related compounds (Fig. 3 C c and d), conjugates and dozens of diarylheptanoids (Fig. 3 C e). Ginger rhizome further contains organic acids, fats, around 50 % sugar, and slimes. [6-19]

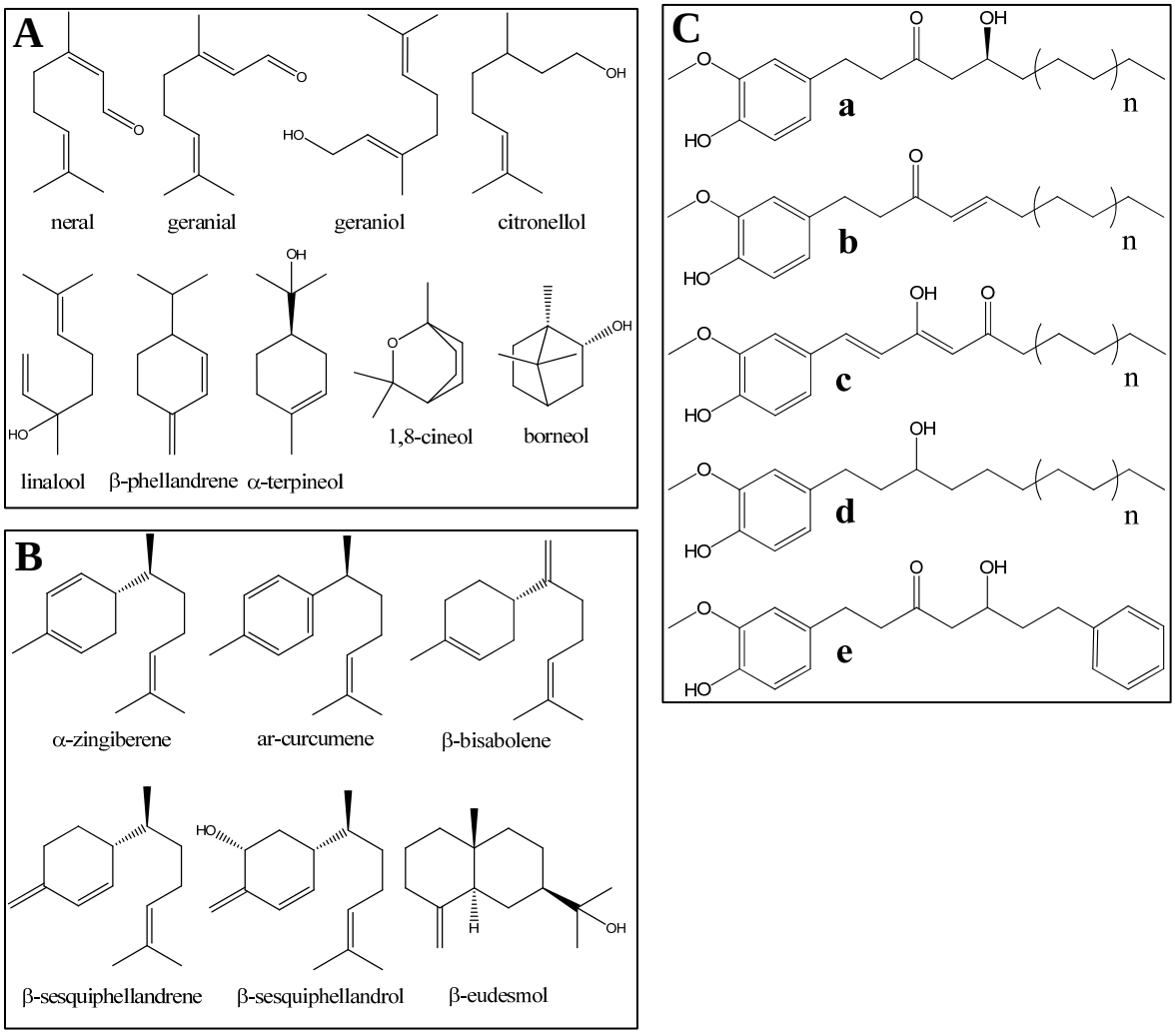


Figure 3: Main secondary metabolites from ginger rhizome.

A Main monoterpenes from the essential oil of ginger rhizome. Their amounts are highly variable between cultivars, harvests, and cultivated areas. **B** Sesquiterpenes from the essential oil of ginger rhizome mainly belong to the bisabolane type. One exception is zingiberol (a mixture of *cis*- and *trans*-β-eudesmol), which is the dominating sesquiterpene and gives ginger rhizome its characteristic odour. The highest sesquiterpene content is found in the 'Jamaican' cultivar [20]. **C**

Four structures of typical phenylpropanoids and an example of a diarylheptanoid of ginger rhizome. a: Gingerol derivatives where n can be 0 to 4. Dominant compounds are 6-, 8-, and 10-gingerol with n = 1, 2, and 3, respectively. b: Shogaol derivatives where n can be 0 to 4. Dominant compounds are 6-, 8-, and 10-shogaol with n = 1, 2, and 3, respectively. c: 1-Dehydrogingerdione derivatives where n usually is 1, 2, or 3. d: Dihydroparadol derivatives with 1-dihydroparadol (n = 1) as the main homologue. Other phenylpropanoids have a decan-3-one (6-paradol), 3,5-dihydroxy-decanone (6-gingerdiol) or 3,5-decadinone (6-gingerdion) side chain. Acetylated and methylated derivatives are also found. e: Diarylheptanoids comprise several structurally related compounds with a 1,7-diarylheptane skeleton often with a hydroxyl or methoxy substitution at position 3, 4 and/or 5 on one or both phenyl rings and/or a keto, hydroxy, and/or acetoxy function at position 3 and/or 5 of the saturated or unsaturated alkyl chain. The example shows 5-hydroxy-1-(4-hydroxy-5-methoxyphenyl)-7-phenyl-3-heptanone.

1.1.4 Traditional and Modern Pharmaceutical Use of Ginger

In China ginger is one of the oldest herbal medicines and dried ginger was first mentioned in the medical book Shen Nong Ben Cao Jing about 2000 years ago; fresh ginger was first listed in Ming Yi Bie Lu and Ben Cao Jing Ji Zhu, two pharmacopoeias written around the year 500 AD [21-23]. In traditional Chinese medicine (TCM) fresh and dried ginger were considered two different commodities; the fresh root is considered a warming and pungent herb that dispels pathogens by inducing sweating. It is supposed to expel cold, to relieve nausea and to 'clear away' toxic matter. The dried root is considered a hot and pungent yang herb which is meant to restore depleted yang and is used against ailments caused by cold, damp weather and is supposed to help against 'cold' pain of the stomach and abdomen, and to be useful for diarrhoea due to 'cold deficiency', cough, and rheumatism. Ginger is also used for the treatment of bleeding disorders and as a digestive aid, against baldness, snakebites, toothache, and respiratory conditions [24].

As for TCM, Ayurveda, the traditional medicine of India, distinguishes between the fresh and dried rhizome which are used similarly to their applications in TCM. Ginger is considered to reduce '*vata*' and '*kapha*', to alter '*pitta*'; it is used to stimulate '*agni*' and alter digestion. Furthermore it is used to block excessive clotting (i.e. heart disease), against arthritis, and to reduce cholesterol, spasms, mucus, and in 'veta' and 'kapha' type fever. In India, ginger juice with honey and sometimes garlic juice is a well known remedy against cough [25].

Unani Tibb, the Arabic system of medicine, classifies fresh ginger as Hot in the third grade and Dry in the first grade, i.e. it is believed to increase the internal heat of metabolism and

digestion to keep the body warm; dried ginger is classified as Hot in the second grade and Dry in the second grade; i.e. it is believed to thin and remove excessive fluids. It is used as a carminative, digestive, and to remove phlegm and obstructions in the vessels. It is further used against diarrhoea, arthritis, toothache, cough, headache, gingivitis, and nervous diseases [26].

In Europe ginger is mainly used for the treatment of motion and sea sickness, nausea, vomiting, postoperative emesis and hyperemesis gravidarum (these indications are the only ones supported by clinical data [27, 28]). Other indications comprise gastrointestinal disorders like dyspepsia, spasms, colic, infections, and peptic ulcers. Furthermore, it is used against rheumatic and muscular disorders [29] and migraine headache [30].

Anti-emetic activity, reduction of nausea, inhibition of ileal contractions, and enhanced gastrointestinal motility by ginger rhizome and phenylpropanoid constituents are well established in *in vivo* experiments and clinical trials. The effects are comparable to those of the standard drugs metoclopramide [31] or domperidon [32]. Indirect antagonism at the 5-HT_3R is likely to contribute to these effects. Noteworthy is the finding that ginger constituents show pro- and anti-kinetic activities depending on the mode of application (e.g. orally or intravenously) or the assayed organ section [33-36]. In animals, emesis evoked by orally applied copper sulphate was inhibited but emesis caused by centrally acting drugs (apomorphine and digitalis) was unaffected thus indicating that ginger constituents have a peripheral activity. This is supported by the fact that ginger is less active in motion sickness than in other cases of nausea [4].

Several small scale clinical trials have shown beneficial effects of ginger or ginger extracts on osteoarthritis [37, 38], gonarthritis [39], rheumatism and musculoskeletal disorders [29, 40]. *In vivo* experiments showed i) anti-inflammatory effects on rat paw and skin edema for ginger extracts [41], ii) an inhibition of PMA-induced inflammation, epidermal ornithine decarboxylase activity, and skin tumor promotion in ICR mice for 6-gingerol [42], and iii) an inhibition of monosodium urate crystal-induced gout in Swiss albino mice equal to a ten fold lower dosage of indometacin for 6-shogaol [43].

In traditional medicine ginger is furthermore used against diabetes and dyslipidaemia. Efficacy in these indications has been confirmed in several animal models and a small placebo controlled clinical trial in humans. Thus, hyperlipidaemic patients showed a significant reduction in triglycerides, cholesterol, low density lipoprotein, and very low density lipoprotein and an increase in high density lipoprotein after consumption of 3 g ginger per day for 45 days [44]. Body weight, glucose, insulin, total cholesterol, LDL

cholesterol, triglycerides, free fatty acids and phospholipids in the serum of rats fed on a high-fat diet were markedly reduced by ginger (HDL cholesterol was unaffected) [45].

In the streptozotocin-induced diabetes models in rats ginger either increased insulin levels and decreased fasting glucose levels [46], or increased serum insulin and glucose tolerance, but had no effect on body weight, fasting blood glucose, blood glycated haemoglobin, liver weight, liver glycogen levels, and serum lipid profiles. These findings are an indication for an insulinotropic rather than a hypoglycaemic effect of ginger [47]. Al-Amin et al. showed lowered serum glucose, cholesterol and triacylglycerol levels after ginger treatment [48]. Bhandari et al. demonstrated an anti-hyperglycaemic effect, lowered serum total cholesterol, triglycerides and increased HDL-cholesterol levels [49], and Ojewole et al. showed a hypoglycaemic effect in untreated (normoglycaemic) and diabetic rats [50]. Fructose-induced elevation in lipid levels, body weight, hyperglycaemia and hyperinsulinaemia in rats were lowered by ginger as well [51]. Differences in reported pharmacological effects may be explained by different dosages of extracts, the use of fresh or dried rhizome and variations in diets.

Based on very recent findings by Heimes et al. the anti-diabetic effects of ginger are linked to the 5-HT_3R antagonistic activity of several ginger constituents [52].

Pharmacokinetic studies that had been done before this thesis was initiated generally included the investigations of pure compounds either *in vitro* [53] or administered by intravenous injection [54]. More recent work (since 2008) also includes studies with orally administered ginger extracts in rats [55-58] and humans [59]. 6-, 8-, and 10-gingerol (6,- 8-, and 10-G) and 6-shogaols (6-S) show a fast resorption, high plasma protein binding, fast blood elimination, a short body half-life (open two-compartment system), and good tissue distribution (brain, heart, lung, spleen, liver, kidney, stomach and small intestine tissues). 6-S undergoes an enzymatic reduction *in vitro*, whereas *in vivo* both 6-S and 6-G are conjugated to glucuronic acid and, at high dose, converted to the sulphate; the conjugates are mainly eliminated via the bile.

Ginger rhizome is on the FDA's 'generally recognized as safe' list [60] and daily intake of several grams fresh plant is not associated with any adverse effects. Latest clinical trials have proven ginger preparations as safe even during pregnancy [27, 28]. In animal studies ginger preparations or pure compounds (mostly 6-gingerol) are often used at high concentrations without toxic effects and there are no known adverse effects reported besides such caused by its pungency.

Data obtained in *in vitro* assays show that phenylpropanoids from ginger may exert toxic effects because they trigger apoptosis in cell lines and/or show unspecific toxicity at concentrations often as low as 10 to 100 μM depending on assay conditions and cell types; toxicity generally increases with lipophilicity of the compounds [61, 62]. As there are no known toxic effects *in vivo* for ginger rhizome and its constituents, it is speculative whether the frequently observed *in vitro* 'anti-cancer' effects are of any pharmacological relevance, even though there is a report about 6-S (but not 6-G!) irreversibly binding to tubulin and thus leading to microtubule damage [63]. The same is true for the mutagenicity of ginger extracts and pure compounds, for which bacterial assays have produced contradictory results; some compounds are pro-, others are anti-mutagenic [64-66]. The potential mutagenicity might be explained by the fact that non-specific anti-bacterial agents and essential oils often show some mutagenic activity in bacteria.

1.1.5 Reported *In Vitro* Effects

Ginger extracts and different constituents (e.g. gingerol-, shogaol-, paradol-, dehydrogingerdion-, and acetylated gingerol-homologues) display an inhibition of PGE_2 production in LPS stimulated promyelotic cell lines at sub-μM concentrations without affecting TNF-α expression [12, 16, 67] and some compounds inhibit 5-HETE biosynthesis in leukocytes at intermediate μM concentrations [68, 69]. Shen et al. showed an inhibition of PGE_2 production in IL-1β, TNF-α, and LPS stimulated sow osteoarthrotic cartilage explants and chondrocytes [70, 71]. Inhibition of prostaglandin and leukotriene (5-HETE) biosynthesis by gingerols and related compounds, and some diarylheptanoids is postulated by Kiuchi et al. [72]. Srivastava et al. reported an inhibition of thromboxane B_2 (TXB_2) biosynthesis and possibly an inhibition of phospholipases, and an amplification of lipoxygenase products in platelets by ginger preparations (with contradictory results for extract fractions of unknown composition) [73, 74]. Even an increase of 6-keto-$PGF_{1\alpha}$ and NO release from rat vascular endothelial cells under hypoxia without effect on TXB_2 is reported by Liu et al. [75]. The underlying molecular mechanisms remain unknown but an inhibition of cyclooxygenases seems obvious as TXB_2 and PGE_2 production serve as surrogate readouts for COX-1 and COX-2 inhibition, respectively [76-78]. At least in some *in vitro* assays a direct inhibition of COX isoenzymes may play a role because gingerols and related compounds inhibit COX-2 function in intact cells [79], 8-paradol and 6-dihydroparadol (6-DHP) inhibit the purified ovine COX-1 isoenzyme with an IC_{50} of 4 and

20 μM, respectively [80], and 10-G, 8-S and 10-S inhibited the COX-2 isoenzyme with an IC_{50} of 32 μM, 17.5 μM, and 7.5 μM, respectively [81]. This concentration range is used in several investigations but will not be achieved *in vivo*. Additionally, hexahydrocurcumin (a minor compound in ginger, but abundant in turmeric), 6-, 8-, 10-G, and 6-S exhibit inhibitory activities on 5-lipoxygenase and prostaglandin synthase isolated from RBL-1 cells [82].

6-DHP inhibits iNOS mRNA expression and NO production in RAW274.7 mouse macrophages (MΦ) at concentrations above 10 μM [83]. 6-G inhibits NO synthesis in activated J774.1 mouse MΦ [84], and 6-G and 6-S inhibit iNOS and COX-2 expression in RAW274.7 mouse MΦ at 10 to 20 μM [85].

6-G inhibits COX-2 expression and the activation of NF-κB and p38 MAPK in PMA treated mouse skin [86].

Another report shows an inhibition of NO, TNF-α, and IL-1β release, iNOS and COX-2 expression, PGE_2 production, phosphorylation of ERK1/2, p38, and JNK, and nuclear translocation of the NF-κB p65 subunit by a ginger hexane extract at 1 and 10 μg/ml in LPS-stimulated BV-2 cells (mouse microglia cell line) [87]. A suppression of nuclear translocation of NF-κB, phosphorylation of IκBα, and membrane translocation of PKC-α in LPS stimulated RAW 264.7 mouse MΦ treated with 20 to 80 μM 6-G was shown by Lee et al. [88] and with 100 μg/ml Zinaxin© ginger extract in synoviocytes activated with TNF-α by Frondoza et al. [89]. Kim et al. demonstrated an inhibitory effect of 6-G on UVB-induced COX-2 expression and NF-κB and caspase activation by blocking the accumulation of intracellular reactive oxygen species [90]. A suppression of NF-κB is postulated by Aggarwal et al. [91] on the basis of COX-1 inhibition published by Nurtjahja-Tjendraputra [80].

One molecular target of gingerols and shogaols is the TRPV1 cation channel, thus explaining the pungency of these compounds and that of ginger rhizome [92-94]. In cultured DRG neurons capsaicin as a standard agonist exerted intracellular Ca^{2+} currents with an EC_{50} of 0.3 ± 0.08 μM whilst 6-G and 8-G showed EC_{50}'s of 56 ± 15 μM and 5.0 ± 0.6 μM, respectively. All compounds were acting as full agonists at the TRPV1 [95].

6-, 8-, 10-gingerol and 6-shogaol display an antagonism at the 5-HT_3 receptor (5-HT_3R) without displacing the selective 5-HT_3R antagonist [^{3}H]GR65630 from the serotonin binding site. This effect includes inhibition of [^{14}C]guanidinium influx through 5-HT_3R channels (1-100 μM) as well as the inhibition of contractions of the guinea pig ileum

induced by the selective agonist SR57227A [35, 96]. In addition, these ginger constituents show weak anti-cholinergic and anti-neurokinergic activities in the guinea-pig ileum [35] and inhibit contractions induced by electrical stimulation and acetylcholine in rat ileum [97].

There are also a few reports on blood coagulation; 6-, 8-G, 8-DHP, and 8-GD inhibit arachidonic acid-induced platelet serotonin release at 57-74 μM and arachidonic acid-induced platelet aggregation at 10-25 μM [98]. 6-G, 8-G, 12-G, 6-S, 8-S, 6-P, 8-P, 6-GD, 6-DHP and a synthetic diarylheptanoid have been reported to inhibit arachidonic acid induced whole blood platelet aggregation [80]. Verma and Bordia showed effects on fibrinolytic activity in a small *in vivo* study [99].

Studies from the late 1980's postulate an activation of the sarcoplasmatic reticulum ATPase from rabbit skeletal and dog cardiac muscles by 6-G at concentrations from 3-30 μM [100, 101].

Phenylpropanoids from ginger show good antioxidant and radical scavenging properties in different assays as can be expected from their chemical structure [19, 102-104].

Most recent investigations proclaim 6-shogaol to be an inhibitor of TLR4 homodimerization and also to interfere with the TRIF-dependent signalling pathway via interaction with TBK1 [105, 106].

1.2 Immune System and Inflammation

The immune system is responsible for the clearance of invading pathogenic organisms, foreign particles or chemical noxa and own faulty cells. It consists of organs, cells, cellular and humoral factors. One important function of the immune system is the tightly regulated use of inflammatory responses with the aim of avoiding or at least limiting tissue damage [107].

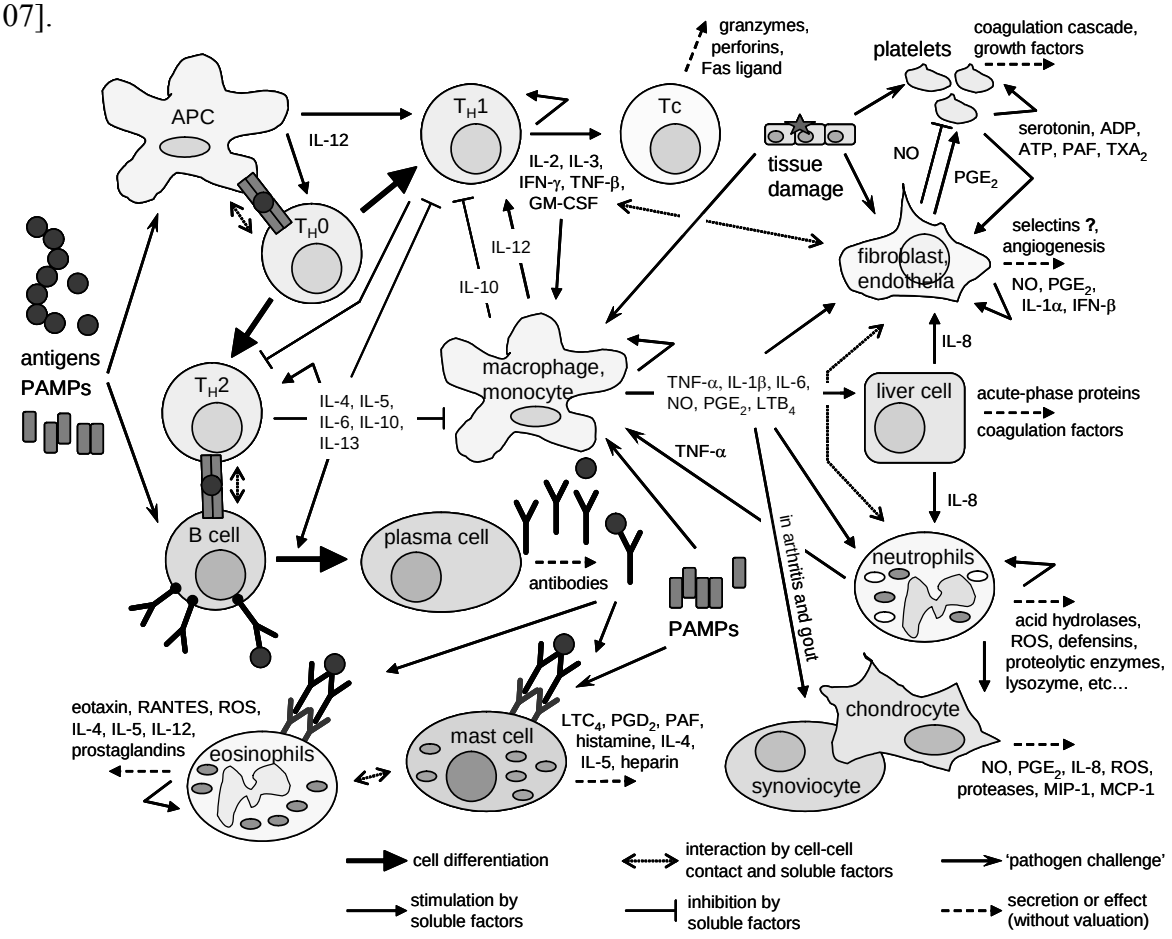


Figure 4: Interplay of immune cells as reaction to a pathogen challenge and tissue damage.
Antigens are processed by antigen presenting cells (APC) and presented to T_H0 cells. These cells then differentiate depending on co-stimulation and surrounding factors either into T_H1 or T_H2 cells. The former trigger a cell mediated immune response by activating MΦ and cytotoxic T cells (Tc), the latter activate B cells leading to a humoral immune response mediated by antibodies. Pathogens and PAMPs bind to Toll-like receptors on/in MΦ, B cells, and/or mast cells and trigger either a 'T_H1' or 'T_H2' response. Tissue damage involves several different cell types depending on cause and organs involved. It usually leads to the activation of monocytes/MΦ and neutrophils with contribution of nearby blood vessels (endothelial cells and fibroblasts). Furthermore, non-lymphatic cells like synoviocytes and chondrocytes are involved in some diseases like arthritis or gout. [108, 109]

Endo- or exogenous noxa lead to a relatively uniform reaction cascade controlled by a complex network of immune cells, the coagulation cascade, blood vessels, the surrounding tissues, and para- and autocrine acting soluble mediators (Fig. 4). However under certain circumstances or in some diseases the inflammatory response is sustained or exaggerated for no apparent beneficial reason for the host. Pharmaceutical, physical, or even surgical interventions might then be advantageous [110] and are indeed practised by mankind from time immemorial.

1.2.1 Innate and Adaptive Immunity

The immune system is divided into two parts; the innate immune system comprises inherited mechanisms and corresponding cell types whilst the adaptive immune system comprises acquired ones. However, the two parts are tightly linked to each other and several cells like monocytes/MΦ or soluble factors like interleukins play important roles in both parts. All cells of the immune system interact with each other and with target cells by cell-cell contact and by a plethora of auto-, para-, and endocrine signalling molecules with cytokines as the major players (Fig. 4) [107].

The innate immune system does not only comprises of immune cell types like granulocytes [111], natural killer cells [112], and MΦ [113], but also physiological and anatomical barriers and proteins and soluble factors such as the complement system, the inflammasome complex, lysozyme, interferons, or acute-phase proteins [107]. This unspecific defence is able to recognize pathogens and foreign matter by molecular patterns (*vide infra*) [114] and fights them by an unspecific, uniform cascade.

The adaptive immune system, on the other hand, is mainly built by antigen-presenting cells (APC), T and B cells, and lymphatic organs. Adaptive defence mechanisms are highly selective and have a high individual variability and specificity. This defence is learned from earlier or ongoing contacts, directed against specific targets (epitopes), and possesses a memory function. It consists of a humoral part; antibodies produced by activated B cells [115] which are regulated by T helper lymphocytes (T_H cells). The cellular part is mainly built by $CD4^+$ T_H cells [116] and $CD8^+$ cytotoxic T cells (Tc) [117].

T_H cells, 'regulatory T cells' (T_{reg}), which mainly maintain immune tolerance, and 'innate natural killer T cells' (NKT cells), which take part in tumor resistance, are involved in the regulation and orchestration of the adaptive immune response [118]. They are assisted by plasmacytoid dendritic cells (DC) which induce T cell anergy and activate T_{reg} [119] and

interdigitate DC, the main source of IL-12. DC regulate activation, anergy, and self tolerance of T cells, cause apoptosis, or generate T_{reg}, thereby preventing autoimmune reactions [120-122]. All these cell types help to direct the immune response towards a cell mediated or a humoral one and are involved in its persistency and its termination.

T_H cells are the main target of cyclosporin A, rapamycin, and basiliximab. These drugs are used to suppress organ rejection after transplantation and occasionally to delay the progression of arthritis and other severe autoimmune diseases.

Regulatory T cells (T_{reg}) are for example involved in arthritis and autoimmunity and would be a great target for future therapies [123-126].

Dendritic cells (DC) consist of several cell types from different origins, most derive from monocytes and express TLR2 and TLR4. The main function of naïve DC is the collection (phagocytosis) and procession of antigens. They are seldom found in the blood stream but are localized at the 'organism-environment interfaces' (skin, mucosa of gastrointestinal tract and airways, pharynx, oesophagus etc.) where they are known as Langerhans cells. A few days after they began to collect antigens, DC cease phagocytosis, migrate to the lymph nodes, and concomitantly differentiate. These matured DC (interdigitating DC) are the most potent APC for T cells and take part in the 'training' and the positive or negative selection of lymphocytes. Dendritic dells are the main targets of vaccines and of the adjuvant 3-*O*-desacyl-4'-monophosphoryl lipid A (adjuvant in the vaccine Cervarix® [127, 128]). Targeting DC to deliberately influence the overall immune response seems promising but is still a theoretic approach.

1.2.1 Cytokines in Inflammation

Cytokines are a large and diverse family of hormone-like, secreted polypeptides. They are multifunctional and exert multiple and often overlapping auto-, para-, and endocrine activities (pleiotropy and redundancy) and are not only indispensable in the regulation of immune responses and cellular communication, but also in the development and homeostasis of the whole organism.

There are three different classification systems for cytokines [129]. According their: i) structure (eight fold families), ii) receptor code (seven types), and iii) function (immunological and non-immunological ones). This last system is most suitable with regard to inflammatory conditions and is the one used in this thesis.

Immunological cytokines are further divided in type 1 and type 2 cytokines. The former enhance cellular immune responses and are also called 'pro-inflammatory', whilst the latter

favour antibody-mediated responses, are able to suppress inflammation, and therefore are called ‘anti-inflammatory’. The most important cytokines are present in low pg/ml concentrations in the blood of healthy donors (IL-8 even reaches the low ng/ml range) and during a diseased state, can be up-regulated a hundred to over a thousand fold within hours to days. Of the best studied pro-inflammatory cytokines interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α) are central in the onset and interleukin 6 and 8 (IL-6 and IL-8) in the maintenance of inflammatory reactions, whilst interleukin 10 (IL-10) serves as the major ‘anti-inflammatory’ cytokine [130].

IL-1 is a family of proteins produced by MΦ, endothelial cells, fibroblasts, and some other cells [131]. Because its member IL-1β plays a key role in this thesis this cytokine will be discussed in detail.

Upon TLR2/-4 ligation circulating monocytes rapidly secrete high amounts of IL-1α and -β. Thus, these cytokines are among the first soluble factors involved in the non-specific immune reaction [132]. They bind to the IL-1 receptors type 1 and 2. The former is constitutively expressed and nearly unchanged in its expression level, whilst the latter is signalling-dead and, together with a soluble IL-1 receptor antagonist, regulates the activity of the two cytokines. IL-1β leads to a subsequent cascade of secondary cytokines triggering a variety of local inflammatory processes (e.g. co-stimulus for T cell activation, up-regulation of E-selectine on and COX-2 expression in endothelial cells, leukocyte extravasation, and oedema formation) and thereby causes several systemic effects (fever, activated thrombocytes, and an increase in circulating neutrophils, acute-phase proteins, and IL-6). Elevated and/or prolonged levels of IL-1β are associated with auto-reactive diseases like rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, and gout [133, 134].

IL-1β is expressed as an inactive 31 kDa pro-form and stored, together with pro-caspase-1, in secretory lysosome-like vesicles. In cases where there is no constitutively activated caspase-1 (also known as IL-1 cleaving enzyme or ICE) nor release of endogenous ATP [135, 136], activation of the inflammasome (e.g. ASC , NALP3) is needed to trigger the autolysis of pro-caspase-1 resulting in the generation of the 17 kDa mature IL-1β [137]. Depending on the cell type ctivation of the inflammasome can be triggered by several TLR ligands like peptidoglycans and muramyl dipeptide, ATP, intracellular pathogens, or uric acid crystals [136, 138-141]. In a second concomitant step the vesicles translocate to the cell membrane, fuse with it, and liberate their content [142, 143]. These pathways are mainly studied in monocytes and monocytic cell lines and are all dependent on several

phospholipases (Fig. 5). Under experimental conditions, expression is usually triggered by LPS and maturation/excretion by $P2X_7$ receptor agonists (e.g. ATP), pore-forming toxins, or the K^+/H^+ ionophore nigericin. All these agonists lead to a drop in intracellular potassium followed by an increase in intracellular calcium due to influx and emptying of intracellular calcium stores [144-146].

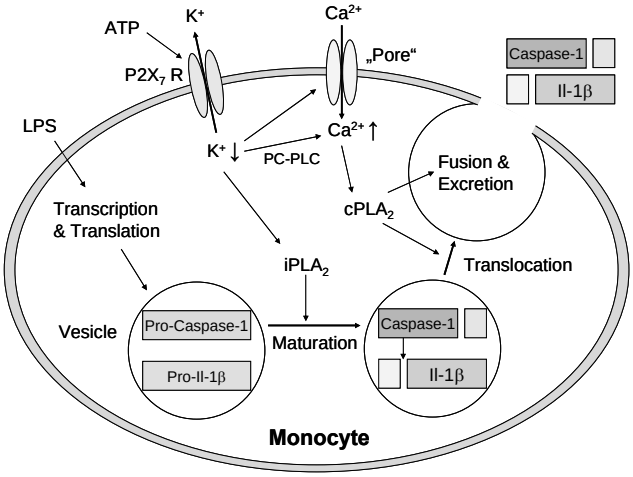


Figure 5: IL-1β secretion in monocytes: a complex network from stimulation to excretion.
Upon *de novo* synthesis (e.g. triggered by LPS), IL-1 β is, unlike other cytokines, stored in vesicles as an inactive pro-form and needs a second stimulus for further processing. The best investigated stimulus is ATP, which leads to a rapid loss of potassium and a subsequent influx of calcium by changes in membrane potential and by a phosphatidylcholine specific PLC (PC-PLC) pathway. Potassium loss activates $iPLA_2$ resulting in the cleavage of pro-caspase-1 and the subsequent cleavage of pro-IL-1 β (maturation). Increased cytosolic calcium activates $cPLA_2$ causing the storage vesicle to translocate to and fuse with the cell membrane for final excretion of mature IL-1 β.

Tumor necrosis factor alpha (TNF-α) has several features in common with IL-1β. It is mainly produced by MΦ, causes local inflammation and generalized effects, and it is expressed as an inactive pro-form to be cleaved before its secretion by the metalloprotease TNF-α cleaving enzyme (TACE). The mature form is secreted early in inflammatory processes as reaction to TLR or IL-1 receptor ligation and subsequently activates several immune cells. It induces cell proliferation, differentiation, apoptosis, leads to cytokine secretion and an increased activity of COX-2, and, together wit IL-6, induces the formation of acute-phase proteins in the liver [147].

Interleukin 6 (IL-6) is also produced mainly by monocytes and MΦ, but also by epithelial and endothelial cells. It stands downstream of TNF-α and IL-1β and is best known for its ability to stimulate lymphocytes and the synthesis of acute-phase proteins in the liver. IL-6 is furthermore involved in the coordination of the transition from innate to adaptive immunity [130, 148].

Interleukin 8 (IL-8) or CXCL8 is secreted by any cell expressing Toll-like receptors but especially by activated MΦ. Furthermore, huge amounts of IL-8 are released into the blood from storage vesicles in liver cells upon stimulation with IL-1β, TNF-α, and IL-6. IL-8 is a so called chemokine (a chemotactic cytokine) and one of the dominant factors attracting neutrophils to the site of inflammation. A wide range of other cell types responds to this cytokine too. IL-8 is activated extracellularly by cathepsin L [149].

Other cytokines important in the area of inflammation and typical for a T_H1 response are IL-2, IL-3, IL-12, and interferons [150]. They further enhance a cell mediated immune response and suppress a T_H2 mediated one. Interleukin 2 is expressed mainly in T_H1 cells upon activation of the T cell receptor complex and regulates differentiation, growth, and survival of cytotoxic T cells and T_{reg} cells, and stimulates cytokine expression in several cell types [151]. IL-2 is also important in the discrimination between self and non-self to prevent auto-immunity. IL-3 is another cytokine typical for T_H1 cells. It leads to the differentiation of multipotent haematopoietic stem cells into myeloid cells and acts as a colony-stimulating factor for this lineage thereby leading to a long-term increase in monocytes/MΦ [152]. In this way, it shifts the immune response towards the innate and cell mediated one. A second dominant colony-stimulating factor is IL-12, which is produced mainly by activated DC and MΦ. It stimulates the differentiation of naïve T cells first into T_H0 and later into T_H1 cells besides activating several other lymphoid cells [109].

Interferons (IFNs) are a class of glycoproteins and are divided in type I, II, and III interferons. IFN-α and IFN–β are secreted in large amounts by plasmacytoid DC to protect against virus infections by halting translation, cleaving host and virus RNA, stimulating the up-regulation of MHC I and II, and activating apoptosis in infected cells [153]. They further activate MΦ and NK cells. IFN expression occurs mainly in response to PAMP receptor activation and therefore takes part in innate immunity [154].

'Anti-inflammatory' cytokines are, for example, Interleukins 4 and 5 (IL-4 and -5) which are secreted by T_H2 cells, eosinophils, and mast cells or interleukin 10 which is produced mainly by monocytes but also by T_H2 and T_{reg} cells. These cytokines are able to shift the T

cell response from a T_H1 to a T_H2 mediated one with the aim of limiting or resolving inflammatory reactions [108, 134]. IL-10 and IL-13 for example inhibit MΦ function and cytokine production by T_H1 cells while enhancing B-cell function. IL-4 leads to differentiation of naïve $CD4^+$ T helper cells into T_H2 cells and to the inhibition of T_H1 cell differentiation and expansion. An overproduction of IL-4 is associated with allergies because it initiates an immunoglobulin class switching to IgE. IL-5 was originally discovered as a colony stimulating factor for eosinophils. In fact, it mainly stimulates B cell expansion and triggers an increased immunoglobulin secretion and is associated with allergic diseases.

Cytokines which apparently do not influence the T cell response are for example interleukin 7 (IL-7) or granulocyte-macrophage colony-stimulating factor (GM-CSF) both acting early in haematopoiesis [155, 156]. IL-7 is a haematopoietic growth factor secreted by several cell types (mainly stromal cells of the red bone marrow and the thymus) except lymphocytes. It causes multipotent haematopoietic stem cells to differentiate into lymphoid progenitor cells whereas GM-CSF is secreted by MΦ, mast cells, T cells, endothelial cells, and fibroblasts and stimulates stem cells to differentiate into the granulocyte and MΦ lineages to satisfy the increasing demand for these cell types during the onset of inflammation.

1.2.3 Pattern Recognition Receptors

An integral part of the innate immunity is the recognition of microbial pathogens and undesired host cells. Differentiation from healthy self (host) molecules is achieved by pattern recognition receptors (PRR), recognizing so called pathogen-associated molecular patterns (PAMPs) and endogenous stress signals or danger-associated molecular patterns (DAMPs) [157]. PAMPs are often high-molecular weight molecules originating from pathogenic but also apathogenic microorganisms like bacteria, viruses, algae, yeasts, and fungi. Until now there is no known PAMP-like structure present in higher plants. PAMPs are highly specific to and constantly associated with these organisms, nearly unchanged during evolution, crucial for the organisms function, and do not occur in organisms expressing corresponding PRR. DAMPs are usually intracellular proteins (like heat-shock proteins) or small molecules (like ATP) which upon tissue injury are released into the extracellular medium where they are often denatured. In a similar manner, tumor DNA can be released after apoptosis to function as a DAMP. Another class of DAMPs are break-

down products of the extracellular matrix generated by tissue injury (hyaluronic acid fragments). There is no distinct receptor class for DAMPs whereas for PAMPs several types of PRR exist and are classified by signalling and endocytic ones. Signalling domain containing PRR, which elicit an intracellular downstream signal and trigger an immune response, are Toll-like receptors (TLR), NOD-like receptors, RIG-I-like-receptors, and some receptors of the C-type lectin/C-type lectin-like superfamily [158-160]. Non-signalling ones are receptors mainly expressed on phagocytes where they support pathogen binding to facilitate endo-and phagocytosis. These latter PRR comprise of some membrane bound β-glucan receptors [160], galectins [161], monocyte mannose receptor [162], and scavenger receptors [163]. Soluble PRR include complement receptors, mannose-binding lectin, serum amyloid A, and C-reactive protein and mainly activate the complement system [164].

1.2.4 Toll-Like Receptors

The most important PRR, with regard to inflammatory reactions, in the immune system are the Toll-like receptors; a protein family where every subtype recognizes a specific set of PAMPs often independent of the host species.

The Toll and Toll-like proteins are a receptor family whose discovery (in the fruit fly *Drosophila melanogaster* [131, 165, 166] and in human [167, 168]) had a great impact on our understanding of the immune system. To date, 10 human TLR family members [169, 170] and several related genes and proteins in other species have been identified.

TLR are a family of transmembrane receptors mainly expressed by monocytes/MΦ and DC. RNA is detectable in many cell types of the immune system whilst protein expression is often contradictory, low, or restricted to specific subtype populations. TLR are part of a rapid defence mechanism of the innate immunity and interact closely with the adaptive immunity. In most cases TLR activation triggers the secretion of pro-inflammatory cytokines like IL-12, TNF-α, and IL-1β and therefore leads to a T_H1 response. Most TLR stimulate and enhance the expression of co-stimulatory molecules on APC and are involved in the pathogenesis of inflammatory diseases [171]. In contrast, TLR7 and TLR9, which are also expressed in pDC, B cells, and the intestinal epithelium, can enhance antibody production and/or exhibit anti-inflammatory activities [172, 173].

Most TLR are functional as dimers and ligand binding leads to the activation of two major pathways [174-176]. The first results in the activation of NF-κB, a master switch for

inflammation, and the second one activates the ‘supporting’ MAP kinases p38 and JNK. The signalling cascade rapidly leads to the expression of pro-inflammatory cytokines (‘early phase’ response) often in a MYD88 and IL-1 receptor associated kinases (IRAK) dependent manner. The MYD88-independent signalling as present in TLR3 and 4 signal transduction acts via different adapter proteins (e.g. TRIF and IRF [177]), is slower, and leads to IFN-β expression (‘late phase’ response). Besides intracellular adapter proteins co-receptors like CD14 or CD36 may modulate TLR signalling.

Among the TLR expressed on the cell surface TLR4 is the most abundant one. It is functional as a heterodimer with MD-2 and signalling can be dependent or independent of the LPS-binding protein CD14. The TLR4/MD-2 complex is a receptor for lipopolysaccharide from Gram-negative and lipoteichoic acids from Gram-positive bacteria, and for host molecules like heat shock proteins, collectins, heparin sulphate, hyaluronic acid fragments, and fibrinogen. TLR4 has not only the broadest ligand spectrum but also the broadest signal transduction. It is expressed as an important recognition molecule on several cells of the myeloid lineage. Epithelial cells in the gastrointestinal mucosa express it too, but the absence of MD-2 expression suppresses a stimulatory effect and therefore protects against inflammatory reactions which would otherwise be elicited by the intestinal flora [178].

TLR2, another cell surface TLR, is expressed on many different immune cells mainly from the myeloid lineage, but also on B cells and T_{reg} cells [179]. It recognizes and internalizes glycolipids from bacteria, peptidoglycanes and lipoproteins/-peptides/-mannans from *Mycoplasma sp.*, lipoteichoic acid from Gram-positive bacteria, the fungal β-glucan zymosan, secondary metabolites from yeasts, viruses, and protista, and a few host molecules. It can build heterodimers with TLR1 and 6 which expands its ligand spectrum [180, 181]. Signal transduction of these three receptors takes place via MYD88 and MAL. TLR1 is located on monocytes/MΦ, dendritic cells, and B cells and recognizes, only as heterodimer with TLR2, triacyl lipopeptides from Gram-positive bacteria and peptidoglycans. TLR6 is expressed on monocytes/MΦ and some cells of the B cell lineage and again, only the heterodimer is functional and recognizes diacyl lipopeptides from *Mycoplasma sp.*

The remaining cell surface receptors comprise TLR5, 10, and 11. The former is expressed on monocytes/MΦ, DC, and, noteworthy, on intestinal epithelial cells and recognizes bacterial flagellin. TLR10 is expressed on monocytes/MΦ and B cells. Although several

TLR10 splice variants are known, function, signal transduction, and ligands are unknown. TLR11 is not expressed as a functional protein in humans [182].

Intracellular subtypes are expressed on the inner membrane side of endosomes. They mainly bind oligodeoxynucleotides and comprise four subtypes. TLR3 is expressed in DC and B cells and recognizes double-stranded RNA from viruses and the synthetic analogue poly(I:C). It is the only TLR with a signal transduction solely via TRIF. TLR7 is expressed in monocytes/MΦ, DC, and B cells and recognizes single-strand uracil- and guanosine-rich RNA and some small synthetic compounds like imidazoquinoline derivatives (imiquimod, resiquinod), loxorubine, or bropirimine. TLR8 is expressed in monocytes/MΦ, some DC, and mast cells and recognizes the same ligands as TLR7. TLR9 is constitutively expressed in pDC and B cells but is inducible in several other immune cells. It binds to the CpG motifs of bacterial and viral unmethylated DNA.

These intracellular receptors are also able to bind modified host nucleosides though with low affinity and insufficient signal transduction and thereby support self tolerance. Nevertheless, in some rare cases inappropriate activation by self molecules (especially RNA- and chromatin-containing immune complexes) can cause autoimmune diseases like systemic lupus erythematosus [173].

1.2.5 Serotonin $_{1A}$ and $_{3}$ Receptors

Serotonin (5-hydroxytryptamine) is a biogenic amine and serves as a neurotransmitter and tissue hormone. Around 90 % of the body's serotonin synthesis, storage, and release take place in enterochromaffin cells. The other part is stored in and released from platelets and to a small amount used in the CNS. In the gut 5-HT serves as a neurotransmitter regulating gastric motility, whereas liberated from platelets it plays an important role in blood coagulation and supports local inflammatory processes. Serotonin is a relevant signalling molecule not only in neuronal but also in immune synapses [183] and plays an important role in innate and adaptive immunity [184]. Several serotonin receptor subtypes are expressed on dendritic cells [185], monocytes, and lymphocytes where they modulate cell migration, cytokine expression, or proliferation [186-188].

Serotonin 1 receptors (5-HT_1R) are $G_{i/0}$ coupled GPCRs widely expressed in the central nervous system (CNS). The most abundant of the five subtypes is 1A (5-HT_{1A}R) which couples to a broad variety of downstream signalling pathways [189-191]. It is involved in mood, emotion, and the modulation of different behavioural responses, including

thermoregulation, sleep, feeding, aggression, and especially anxiety [192-197]. The therapeutically used azapirone anxiolytics buspirone (Buspar®), gepirone (Gepiron®), and tandospirone (Sediel®) exert their anxiolytic activity by partial agonism at the 5-HT_{1A}R [198] and may be equally effective in the therapy of depression as the selective serotonin reuptake inhibitors fluoxetine (Prozac®) and citalopram (Seropram®) [199]. Furthermore it is one of the target receptors of several antidepressant drugs [200] and is partially involved in migraine aetiology [201]. Furthermore the 5-HT_1R is expressed on lymphocytes where it alters proliferation, activates the ERK kinase and NF-κB pathways, regulates survival, and modulates immune processes [184].

The serotonin 3 receptor (5-HT_3R), unlike the other 5-HT receptors, is a ligand-gated and non-selective cation channel. It is expressed on leukocytes, enterochromaffin cells, and central and peripheral neurons. It plays a major role in gastrointestinal motility and secretion and in the central vomiting reflex [202]. 5-HT_3R antagonists (e.g. granisetron, ondansetron, and tropisetron) and mixed-type 5-HT_4R agonists (tegaserod and metoclopramide) are used against postoperative and chemotherapy-induced emesis, nausea, gastric reflux, constipation, and/or irritable bow syndrome.

1.2.6 Phospholipases A2

Phospholipases (PL) are enzymes hydrolyzing the ester bonds of phospholipids and are classified according to their cleavage sites (Fig. 6) into PLA_1 (involved mainly in lipid metabolism), PLA_2 (*vide infra*), PLB (metabolism of lysophospholipids), PLC (signal transduction by second messenger generation), and PLD (metabolism and signal transduction). Autotaxin has the same cleavage site as PLD, but acts on lysophospholipids.

Phospholipases A_2 are classified into five types: secreted PLA_2 ($sPLA_2$), cytosolic PLA_2 ($cPLA_2$), calcium-independent PLA_2 ($iPLA_2$), platelet-activating factor acetylhydrolases (PAF-AH, also known as lipoprotein-associated phospholipase A_2 or Lp-PLA_2), and lysosomal PLA_2. Modern nomenclature uses the group numbering system by Edward A. Dennis [203] which has its origin in the century old definition of snake venom $sPLA_2$. Several $sPLA_2$ were intensively studied and recognized as active principles of snake and bee venoms and of mammalian pancreatic juice already in the early 1900s. The first non-secreted PLA_2 was discovered in the 1980s and in the 1990s the first human $sPLA_2$ and $cPLA_2$ were sequenced [203]. Their importance beyond lipid metabolism, especially of the $iPLA_2$ type, has only been recognized during the last twenty years and our knowledge on

the role of these PLs are still limited. Several alternative names are in use, many of the described effects are not attributed to a certain subtypes, the iPLA$_2$ groups are often neglected, and many inhibitors used for *in vitro* studies are not as selective as they were proposed to be; therefore many earlier findings have to be re-visited or even revised [204].

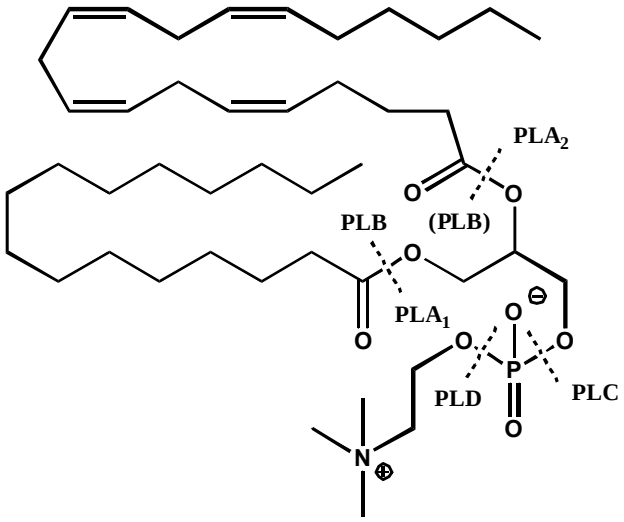


Figure 6: Structure of a phospholipid (1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine) and the cleavage sites of phospholipases.

PLA$_1$ enzymes cleave the sn-1 position and are involved mainly in lipid metabolism. PLA$_2$ isoenzymes cleave the sn-2 position of different phospholipids. PLB are enzymes which can cleave the sn-1 and sn-2 position but usually cleave the sn-1 position of lysophospholipids. PLC cleaves at the sn-3 position. The mammalian isoforms phosphoinositide PLC have a preference for polyunsaturated phosphatidylinositol phosphates. The two mammalian PLD isoenzymes cleave off the choline head-group of monounsaturated or saturated phospholipids whilst autotaxin acts on lysophospholipids.

The distinct PLA$_2$ types have no sequence homology, different catalytic mechanisms, and unequal substrate and calcium requirements. The only thing in common is that they hydrolyse the *sn*-2 ester bond of diacyl-phospholipids to generate lysophospholipids and free fatty acids.

Both products are precursors for a variety of different mediators of physiological and pathological processes. For exampke, lysophospholipids are transformed into platelet activating factor by acetyltransferases [205] while arachidonic acid is metabolized by cyclo- and lipoxygenases into eicosanoids [206, 207]. Besides regulatory functions in lipid digestion (sPLA$_2$ IB), lipid metabolism, and cell membrane bilayer remodelling (mainly iPLA$_2$ VIA-1 [208, 209]) as housekeeping functions, phospholipases A$_2$ are also involved in

inter- and intracellular signalling and calcium signal transduction (mainly $iPLA_2$ VIA-2) [210-215]. Furthermore they are involved in apoptosis [216-218], proliferation and maturation [219, 220], glucose-induced insulin secretion [221], and, most important for the topic of this thesis, play crucial roles in acute [222] and chronic inflammatory diseases (e.g. $sPLA_2$ IIA, $cPLA_2$ IV) [223-226]. PLA_2 are also involved in IL-1β [142] and IL-18 [227] maturation ($iPLA_2$ VI) and secretion ($cPLA_2$ IV), though the former has recently been put into question [228]. Noteworthy is the bactericidal activity of $sPLA_2$ IIA, which is present in tears, intestinal lumen, inflammatory exudates etc. [229] and which may play a role in innate immunity.

The PLA_2 isoforms are regulated by different mechanisms: $sPLA_2$ require calcium at mM concentration in the active site and for substrate binding. In addition to the preference for negatively charged phospholipid surfaces and a strong interfacial activation [230] no regulatory mechanisms after secretion are needed for enzyme activation, though there are several known molecules (e.g. M-type lectins in mice) binding $sPLA_2$ and regulating their activities [231, 232]. $cPLA_2$ require calcium at a μM concentration for substrate binding and membrane translocation. $cPLA_2$ IV are the sole PLA_2 with a preference for arachidonic acid at the *sn*-2 position of phospholipids [233-236] and $cPLA_2$ IVA is even thought to be the main source of free arachidonic acid *in vivo*. $cPLA_2$ activity is regulated by the intracellular calcium concentration, subcellular localisation, phosphorylation state, protein-protein interactions, and *de novo* synthesis. It is furthermore enhanced by binding to ceramide-1-phosphate and phosphatidylinositol bis- and trisphosphates, whereas cleavage by caspases-3 and -8 inactivates these enzymes [237]. $iPLA_2$ neither require calcium for substrate binding nor for catalysis, their activities are regulated by subcellular localization. The activity of the multimeric group VI enzymes is modulated by competition with lipase-dead splice variants [238, 239]. *In vitro,* ATP is known to prolong $iPLA_2$ activity and short chain phosphatidylcholines exert an interfacial activation. However, the physiological relevance of these effects has not yet been shown (reviewed in; $sPLA_2$: [240], $cPLA_2$: [237, 241], $iPLA_2$: [204, 242], general reviews: [243-246]).

1.2.7 MAP Kinases

Mitogen-activated protein (MAP) kinase signalling involves a phylogenetically well conserved cascade of three to five serine/threonine-specific protein kinases. The MAP kinase pathways alter differentiation, proliferation, cell survival, apoptosis, and cytokine production within minutes to several hours [247, 248].

The three dominant MAP kinase pathways are the ERK1/2, JNK, and p38 pathway.

The extracellular signal-regulated kinases ERK1 and ERK2 are activated by extracellular growth-factors (e.g. EGF, PDGF, or FGF), TCR/CD3 and TCR/CD28 ligation, UV irradiation, and trophic factors. They mainly regulate gene expression related to growth, proliferation, differentiation, and cell cycle control.

The c-Jun N-terminal kinase (JNK) pathway comprises the three JNK isoforms and their kinases and is activated by stress-related factors like cytokines (TNF-α, IL-1β, or TGFβ), lipopolysaccharide, UV irradiation, or heat and osmotic shock. The JNK pathway regulates differentiation, proliferation, apoptosis, and cytokine expression (e.g. IL-8, GM-CSF, and RANTES) especially in lymphocytes and under inflammatory conditions [248].

The p38 pathway, named after four p38 MAP kinase isoforms, is activated by the same factors as the JNK pathway, but also by FAS ligand, growth factors, TLR ligation, and a broader set of inflammatory cytokines leading to similar effects as the JNK pathway. P38 isoforms are on one hand important mediators in the progression of inflammatory processes and their inhibition is a possible mechanistic approach for the development of new therapies of inflammatory diseases [249]. On the other hand p38-α is also involved in iT_{reg} function leading to T_H1 cell anergy [250, 251].

1.2.8 Fighting Inflammation, An Ongoing Task

Inflammatory disorders are frequent complaints and there is a broad spectrum of medication and therapy available. The most often used remedies in the First World are ‘non-steroidal anti-inflammatory drugs’ (NSAIDs) acting downstream in the early inflammation cascade by interfering with eicosanoid synthesis. The therapeutic effect is mainly caused by the inhibition of COX-2 which transforms arachidonic acid into the endoperoxide prostaglandin G_2 (PGG_2). In short-term therapy NSAIDs are well tolerated, show moderate analgesic and antiphlogistic activities but tend to exert activity-linked side-effects [252, 253] because PGG_2 serves not only as substrate for the synthesis of

prostaglandins, causative agents of inflammation, but also to counteract thromboxanes and prostacyclins.

Good antiphlogistic activities are achieved by anti-inflammatory glucocorticoids. They affect all nucleated cells and hence may lead to a manifold of adverse effects when used systemically over long time (e.g. exogenous Cushing´s syndrome [254]). Therefore glucocorticoids are used only in severe and/or acute diseases or as topical drugs (ointments or asthma inhalers).

For the treatment of severe and chronic inflammation (e.g. rheumatoid arthritis, Crohn's disease, or inflammatory bowel disease) 'disease modifying anti-rheumatic drugs' (DMARDs) are frequently used. These drugs slow down disease progression by partial immunosuppression, thereby exhibiting an indirect anti-inflammatory activity [255]. These therapies are restricted to a few months due to adverse effects.

The latest approved class of anti-inflammatory drugs is termed 'biologicals', proteins directed mainly against cytokines (often monokines), often upstream targets in the inflammation cascade. Market leaders are the IL-1 receptor antagonist anakinra (Kineret®), the TNF receptor-p75 Fc fusion protein etanercept (Enbrel®), and the anti-human TNF-α monoclonal antibodies infliximab and adalimumab (Remicade® and Humira®, respectively). These compounds exert high potency but, besides common disadvantages of most bigger therapeutic proteins, show other unique side effects [256-258]. Also B cells as targets in inflammatory diseases gain increasing interest. Rituximab (MabThera®) is efficient against rheumatoid arthritis [259] whilst others seem promising against systemic lupus erythematosus [260].

Attempts to treat inflammation by the selective inhibition of single pathways, enzymes, or receptors in the inflammation cascade often failed due to the lack of effectiveness [261]. One of the few well established drugs are H1 receptor antagonists which are useful in allergy related disorders [262, 263]. The 5-HT_3R antagonist tropisetron [264] seems to be promising whilst for other serotonin receptors, even though involved in inflammation, pain, and vasoactive complaints, there are no ligands approved for these indications yet. The need for stronger, more selective, and better tolerated drugs suitable for long-term use led to a lively search for small molecules targeting specific biosynthetic or signalling pathways of pro-inflammatory cytokines. One of the best studied potential targets in inflammation is NF-κB. This nuclear factor is of great clinical significance as it is constantly activated in many cancer cells, it plays major roles in the onset of inflammation, and furthermore has an

increased activity in several inflammatory diseases [265]. Noteworthy is that several medicinal herbs and other plants which show *in vitro* anti-inflammatory or cytotoxic activities appear to inhibit NF-κB activation as mode of action (p. ex. [266]). Nevertheless, so far there is no approved drug directly and selectively targeting NF-κB.

Other foci in research are IL-1β converting enzyme (ICE) [267], TNF-α converting enzyme (TACE) [268], or p38 [261] and other MAP kinases [269]. Further targets are different classes of phospholipases A_2 [270], some of which seem to be inhibited as side-effect by already approved drugs like quinacrine [271]. Hundreds of potent inhibitory small molecules of the aforementioned enzymes were found *in vitro*,but, unfortunately, none of these has reached the market [272] and therefore the search is ongoing. In 2010 Glaxo-Smith-Kline reached clinical phase III with the Lp-PLA_2 inhibitor darapladib [273] and phase II with the p38 inhibitor losmapimod, whereas several pharmaceutical companies have more or less selective MEK1/2 inhibitors in phase I.

1.2.9 Inflammation Assays Using Whole Blood

As a result of the complexity of inflammatory processes, animals clearly represent the model of choice, even though they have several disadvantages like being laborious, time-consuming, or lacking inter-species transferability (like in rheumatoid arthritis [274]). Primary cells and cell lines, otherwise common alternatives, are easy to handle, better defined, and exhibit less inter-assay variations, but seldom show the same behaviour as in a physiological network. A compromise between these two model systems is (human) whole blood, a mixture of interacting cell types in a physiological medium [275].

Whole blood for *in vitro* investigations is usually used to isolate specific cell populations and may serve as an additional tool in screening chemical libraries or new compounds. Nevertheless, publications dealing with whole blood assays are sparse even though whole blood assays not only reduce the time for manipulation (in contrast to purified single cell populations) but also most closely approximate the condition of circulating cells *in vivo*. Modern purification techniques like magnetic bead based isolation or density gradient centrifugation using non-ionic and isoosmotic iodixanol gradients are much more gentle than older procedures but none the less require several manipulation steps which may alter cell function. Moreover, artificial culture media are usually made of heterologous serum containing cytokines, growth factors, and hormones of foreign species with an unpredictable ability to influence cell culture behaviour. In contrast, un-manipulated whole

blood contains physiological concentrations of factors needed for regular cell function [276]. Another advantage of whole blood compared to isolated cell populations is the ability of the involved cell types to interact with each other resulting in signal modulation, amplification, or attenuation. It furthermore allows synchronically distinguishing and quantifying the involved cell types for example by detecting stimulus-induced cytokines expression [275, 277-279].

1.3 Arabinogalactan-Proteins

Whilst investigating hydro-alcoholic extracts of ginger in whole blood we found that these extracts elicit a strong expression of monokines with a pattern similar to the one caused by LPS stimulation. TLC revealed the presence of polysaccharides and proteins in these extracts and their absence in 'non-stimulatory' extracts obtained with supercritical CO_2. Aqueous vegetable extracts from different plant species were used for comparison yielding the same results. Moreover, residual endotoxin contamination did not correlate with these effects, thus suggesting a PAMP-like effect induced by the plant extracts. Polysaccharides and peptidoglycans are well known ligands for several pattern recognition receptors (for example β-glucan receptors and TLR2, respectively) and, together with arabinogalactan-proteins (AGPs), have been reported to stimulate monocytes/MΦ [280, 281]. Preliminary *in vitro* binding studies by Thude et al. showed binding to monocytes, lymphocytes, and granulocytes in a CD4 and CD8-independent manner [282]. Deprivation of vegetable extracts of AGPs reduced their ability to stimulate cytokine expression whilst AGP enrichment enhanced it. Importantly, these effects have been attributed only to medicinal plants so far. As aqueous plant extracts are often described as 'immunostimulatory' in cellular assays the above findings triggered a more detailed investigation into the cellular effects of AGPs.

1.3.1 Origin and Biological Function of AGPs

AGPs are a class of high-molecular weight proteoglycans that are widely distributed in all plants where they play important roles during plant development and in growth regulation [283-285]. They are localized in cytoplasmic organelles, cell walls, plasma membranes and are part of several plant gums and mucilages. AGPs usually consist of a small linear core protein and more than 90 % *O*-linked glycan side chains built of a galactosyl-rich framework with β-(1,3)-, β -(1,6)-, and β -(1,3,6)-linkages and terminal arabinose residues (Fig. 7) and minor sugars such as glucuronic acid and rhamnose. 'Classical' AGPs are rich in hydroxyproline, Ala, Ser, Tyr, and Gly. Membrane-bound AGPs additionally contain a glycosylphosphatidylinositol (GPI) anchor attached to the polypeptide backbone [283-285].

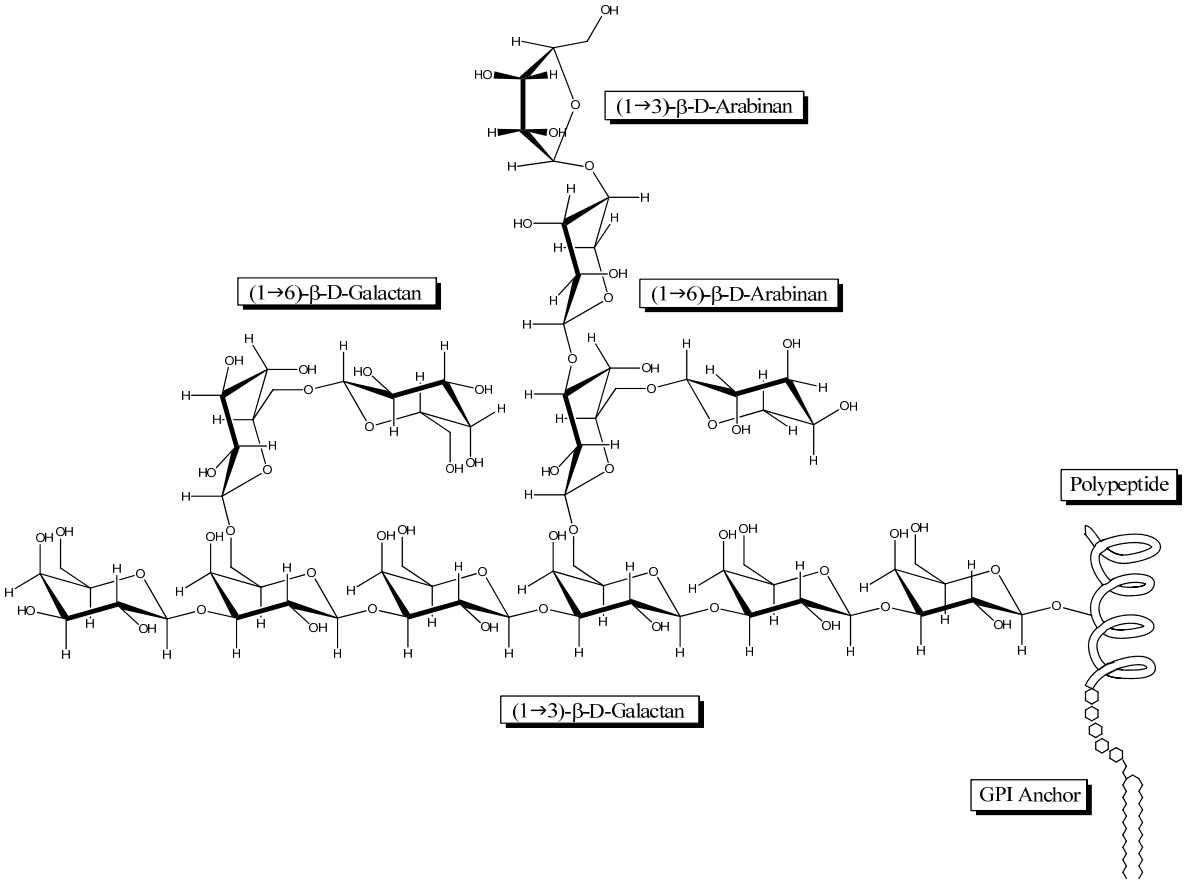


Figure 7: Simplified structure of a membrane bound arabinogalactan-protein.

AGPs are small polypeptides being highly *O*-substituted with branched galactans bearing terminal arabinans. Native AGPs have a C-terminal glycosylphosphatidylinositol (GPI) anchor and thus are membrane bound. A PI specific phospholipase C can cleave this GPI anchor to liberate the AGP into the cytosol or the extracellular matrix.

A common feature of many AGPs is that they can be isolated by co-precipitation with Yariv's reagent, a diazo-glucosly derivative (Fig. 8) [286]. Yariv's reagent is extensively used in AGP quantification and in functional characterisation in plant cell cultures. The first isolated AGPs were precipitated with it, but the underlying mechanism is still unclear. The interaction of Yariv's reagent with AGPs is reversible, depends on the structure and anomeric configuration of the phenyl-linked saccharide moiety, and is sensitive to ions and solvents [287-289].

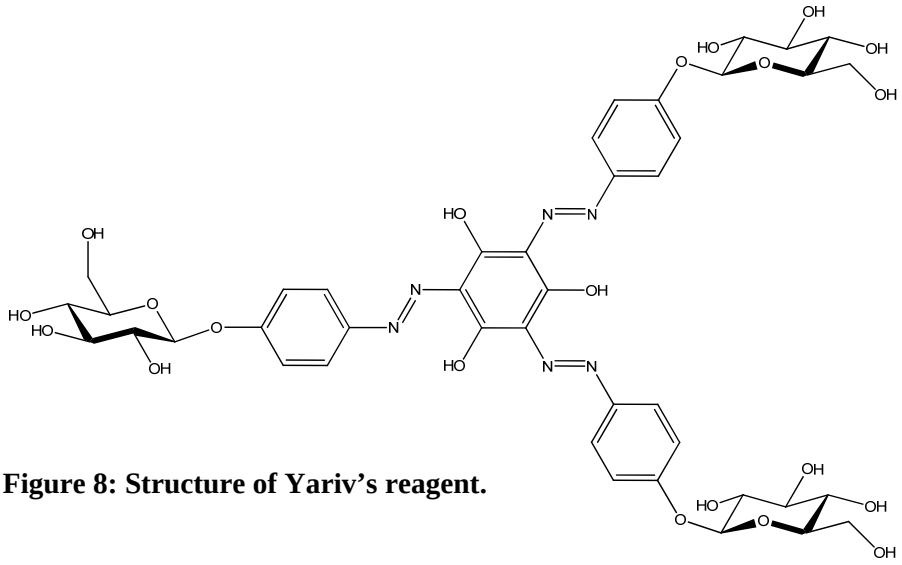


Figure 8: Structure of Yariv's reagent.

1.3.2 Effects on Animals

AGPs are known to stimulate phagocytosis and TNF-α release in monocytes/MΦ [290], lymphocyte proliferation, IgM production and cytokine release [281, 291] *in vitro*. AGPs from the aerial parts of *Echinacea purpurea* have also been shown to stimulate the classical and alternative pathways of complement activation in human pooled serum [292].

No pharmacokinetic data are currently available either for orally administered AGPs in humans or animals, or in *in vitro* absorption models.

Ex vivo studies with Peyer's patch cells isolated from mice after oral treatment with AGP-containing extracts showed an increased reaction to these extracts and to LPS, thus indicating that AGPs might be orally bio-available [293]. Several plant arabinogalactans and other polysaccharides show an activation of MΦ (species independent) too [280] and this has led to the hypothesis that they might bind to pattern recognition molecules like TLR4 [294], CD14, complement receptor, scavenger receptor, dectin-1, or mannose receptor [295]; this could then also be true for AGPs.

1.3.3 The 'Immunostimulation' Theory

AGPs have been termed 'immunostimulating' *in vivo* [296], but usually the corresponding studies neglect the fact that such large molecules are unlikely to be bioavailable. Even though AGPs are present in every higher plant, they are often claimed to be at least partially responsible for the pharmacological activities of specific medicinal plants [295-302]. It should also be noted that the expression 'immunostimulation' in the context of medicinal plant preparations is somewhat confusing, because stimulating the immune system is not necessarily curative or healing and could as well be an undirected and uncoordinated response or a septic shock-like condition. A more suitable expression is 'immunomodulation', a term used for example for the drug imiquimod (Aldara™) or preparations of attenuated ovine parapoxviruses (Zylexis®, a so called 'para-immunity inducer') [303] and generally applied to drugs which alter the immune reaction in an indirect manner. For example, imiquimod binds to the TLR7 [174] and processed parapoxvirus dsDNA binds to TLR9 in MΦ and DC which in turn activate T_H1 cells to express interferons [153]. This results in up-regulated MHC I presentation on surrounding cells and the elimination of MHC I-lacking cells by cytotoxic T cells and natural killer cells. Rather, AGPs exert cytokine patterns that are typical for a generalized inflammatory innate immune response which bears the risk of possibly causing severe undesired effects in healthy subjects too.

If there are pharmacologically relevant systemic effects for orally administered AGPs, they might be achieved by the following mechanisms. If not digested, AGPs are likely to be collected by microfold cells (M cells) [304] and histiocytes mainly in Peyer's patches and other parts of the gut-associated lymphoid tissue ('GALT', which belongs to the reticuloendothelial system) in the lamina propria of the small intestines. M cells have the ability to collect large antigens from the gastrointestinal lumen and deliver them via transcytosis to their basolateral side and to antigen presenting cells like dendritic cells, MΦ, and γδ T cells. The γδ T cells are a small sub-population of T lymphocytes mainly located in the intestinal mucosa with a special T cell receptor subtype. These cells are not HMG restricted and seem to recognize whole proteins or, if freely circulating in the blood, the microbial metabolite HMB-PP. γδ T show a similar effector function as αβ T cells, but furthermore function as potent APC, optimal requirements for the aforementioned *ex vivo* studies with Peyer's patch cells [293].

Additionally, the phagocytes located beneath the gut epithelium can penetrate the latter with their pseudopodes to directly collect pathogenic microorganisms and other antigens in the intestinal lumen [304]. This builds a first line of defence against invading microorganisms and sustains the mucosal immunity. While not leading to quantitative uptake and systemic bioavailability of unprocessed macromolecules, this mechanism also results in 'resorption' of large particles and microorganisms which are digested and broken down to present processed small antigens to other immune cells. In that way AGPs under certain circumstances (e.g. laboratory/assay conditions) may stimulate immune cells in the 'GALT' and subsequently lead to a systemic 'immunomodulation' by stimulating the expression of corresponding antibodies or pro-inflammatory cytokines. However, from an evolutionary and physiological point of view, a systemic pro-inflammatory reaction against omnipresent nutritional constituents seems inappropriate. Immune reactions against intestinal microorganisms are common, but are strictly regulated to protect the organism from overreaction to this constant challenge. Preliminary data show that nutritional constituents ("prebiotics") can interfere with intestinal microorganisms and in that way may modulate the immune response [305].

2. Aim of Thesis

In this thesis the immunomodulatory principles in ginger rhizome were assessed in different biological assays *in vitro* with the aim to improve the current understanding of the reported anti-inflammatory therapeutic effects of ginger. To that aim, the type of active compounds, their cellular targets, and the mode of action on a molecular level were investigated.

As a basis for the implementation of these studies a validated and robust human whole blood assay was established, which allows not only the characterization of anti-inflammatory activities of ginger but also of other herbal preparations, drugs against inflammatory diseases, and selective inhibitors of contributing signalling pathways. In a first phase of the project ginger extracts and selected compounds were assayed for their modulation of stimulated cytokine expression in freshly drawn human whole blood. Based on effects found with ginger extracts and their constituents in the whole blood assay the underlying molecular mechanisms were investigated in more detail in experiments using cell lines and and isolated human leukocytes. A differentiated insight into the action of ginger on distinct cell types and their individual reaction to ginger preparations could thus be gained, leading to the discovery of PLA_2 inhibiting compounds in ginger rhizome.

At the beginning of this work data on absorption for ginger and its constituents were lacking and therefore a resorption model was set up. To estimate the resorption of ginger extracts and individual constituents, they were examined for their passage through a Caco-2 monolayer. Additionally, a model linking whole blood experiments to the Caco-2 model could be established in which the buffer on the basolateral side of the Caco-2 membrane was replaced by blood. In contrast to common assay procedures the modulation of basolateral cytokine expression by permeating compounds was used as a read-out and not the concentration of the individual compounds as such.

Preliminary data and traditional indications for ginger rhizome served as basis for four working hypotheses that were investigated experimentally. This included the hypotheses that ginger constituents i) modulate the peripheral serotonergic system, ii) interfere with the Ca^{2+}-mediated activation of lymphocytes, iii) inhibit p38 MAP kinase (as postulated by Kim et al. [86]), and iv) that arabinogalactan-proteins act as Toll-like receptor agonists. The latter hypothesis emerged from a coincidental finding. The first three hypotheses relate to putative mechanisms of action of ginger constituents, which are crucial in the course of

inflammatory disease progression, and could explain many of the traditional applications and postulated effects of ginger [184, 306].

Ginger preparations and pure compounds were tested on relevant serotonin receptors (5-HTR). The traditional use of ginger implies a possible involvement of the subtypes 5-HT_{1A}, 5-HT_{2A}, and 5-HT_3 at which radioligand-displacement by ginger extracts and extract fractions were to be investigated. The isolation of receptor-interacting compounds from a whole extract was achieved by an iterative process of fractionation, displacement assays, and re-fractionation of active fractions. The structures of pure compounds were elucidated and binding affinities at the corresponding receptors determined. In a subsequent step functional assays provided the necessary tools to determine whether these constituents act as agonists, silent antagonists, or inverse agonists.

A ginger extract and its main constituents were tested for their ability to modulate the phosphorylation state of MAP kinase in isolated human lymphocytes and calcium signalling in Jurkat cells.

Initially, AGP contamination caused contradictory results when investigating ginger extracts. Following up on these findings, the effects of AGPs were investigated more closely, as AGPs are omnipresent in aqueous plant preparations and might be responsible for what have been termed ‘immunostimulatory activities’ of several medical plants. The AGP content of a set of plants from different genera was determined, their AGPs were isolated and tested for their ability to trigger immunoreactions in human whole blood and isolated cell types. In particular, the question was addressed whether Toll-like receptors may be the molecular target of AGPs through cytokine measurements, binding studies, and experiments with knock-out mice.

3. Materials and Methods

3.1 Chemicals

Chemical	*Supplier*
1α,25-Dihydroxycholecalciferol (Calcitriol)	Sigma-Aldrich Chemie GmbH
1,2-Distearoyl-*sn*-glycero-3-phosphate sodium salt	Avanti Polar Lipids, Inc.
1,4-Dioxane, reagent grade	Scharlau SA
1,4-Dithio-DL-threitol (DTT), high purity	GERBU Biochemicals GmbH
1-Hexadecyl-2-arachidonolythio-2-deoxy-*sn*-glycero-3-phosphorylcholine (thio-PC)	Cayman Chemicals
2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid (HEPES)	Sigma-Aldrich Chemie GmbH
2,4'-Dibromoacetophenone (DBA)	Fluka Chemie AG
2-Mercaptoethanol	Fluka Chemie AG
2-Propanol (isopropanol)	Scharlau SA
[^{3}H]-8-Hydroxy-2-(dipropylamino)tetralin hydrobromide, 1 mCi/ml ([^{3}H]-DPAT)	PerkinElmer
[^{3}H]-GR65630, 1 mCi/ml, 77.2 Ci/mmol	PerkinElmer
[^{35}S]-Guanosine 5'-O-[gamma-thio]triphosphate di-lithium salt, 1 mCi/ml, 1250 Ci/mmol ([^{35}S]-GTPγS)	PerkinElmer
3-(N-Morpholino)-propanesulfonic acid (MOPS)	Omnilab, Sigma-Aldrich
5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB)	Fluka Chemie AG
E-6-(Bromoethylene)tetrahydro-3-(1-naphthyl)-2H-pyran-2-one (Bromoenol lactone or BEL)	Sigma-Aldrich Chemie GmbH
6-Gingerol (6-G), authentic standard	ChromaDex Inc.
6-Shogaol (6-S), authentic standard	ChromaDex Inc.
8-Gingerol (8-G), authentic standard	ChromaDex Inc.
8-Hydroxy-2-(dipropylamino)tetralin-hydrobromide (DPAT)	Sigma-Aldrich Chemie GmbH
10-Gingerol (10-G), authentic standard	ChromaDex Inc.
Acetic acid 100 % (glacial acetic acid)	Fluka Chemie AG
Acetone	Fluka Chemie AG
Acetonitrile LiChrosolv	Merck (Schweiz) AG
Acridine Orange hydrochloride hydrate	Fluka Chemie AG
Adenosine 5'-O-triphosphate disodium salt (ATP)	Fluka Chemie AG
Ammonia ~30 % (NH_3)	Fluka Chemie AG
Ammonium heptamolybdate tetrahydrate	Sigma-Aldrich Chemie GmbH
Amphotericin B solution 250 µg/ml (A2942)	Sigma-Aldrich Chemie GmbH
Aniline	Fluka Chemie AG
p-Anisaldehyde (4-methoxy benzaldehyde)	Fluka Chemie AG

Antioxidant NuPAGE® (20X)	NOVEX® Invitrogen
Arachidonic acid	Tocris Biosciences
Ascorbic acid	Fluka Chemie AG
Bovine serum albumin fraction V (BSA)	Fluka Chemie AG
n-Butanol	Scharlau SA
Caesium carbonate (Cs_2CO_3)	Sigma-Aldrich Chemie GmbH
Calcium chloride ($CaCl_2$)	Merck KGaA
Candida albicans	ATCC
Cerium(IV) sulphate	Fluka Chemie AG
Cetirizin dihydrochloride *BioChemika*	Sigma-Aldrich Chemie GmbH
Chloroform ($CHCl_3$)	Scharlau SA
Chloroform-d	Sigma-Aldrich Chemie GmbH
Curcumin	Carl Roth®
Cyclohexane (in house redistilled)	Fluka Chemie AG
Cyclosporin A *BioChemika*	Sigma-Aldrich Chemie GmbH
Deuterium oxide (heavy water, D_2O)	Sigma-Aldrich Chemie GmbH
Dexamethasone 21-phosphate disodium salt	Sigma-Aldrich Chemie GmbH
Dichloromethane (DCM)	Scharlau SA
Diclofenac sodium salt	Sigma-Aldrich Chemie GmbH
Diethyl ether (in house redistilled)	Fluka Chemie AG
Dimethylsulfoxide (DMSO)	Sigma-Aldrich Chemie GmbH
Diphenylamine	Fluka Chemie AG
Disodium hydrogen phosphate (Na_2HPO_4)	Fluka Chemie AG
Dulbecco's Modified Eagle's Medium	GIBCO® Invitrogen
ECL Plus Western Blotting Detection Reagent	Amersham Biosciences
Ephedrine hydrochloride	Fluka Chemie AG
Ethylenediaminetetraacetic acid (EDTA)	Fluka Chemie AG
Ethylene glycol tetraacetic acid (EGTA)	Fluka Chemie AG
Ethanol absolute, 99 % (EtOH abs.)	Fluka Chemie AG
Ethanol, 96 % (EtOH)	Fluka Chemie AG
Ethyl acetate (in house redistilled)	Fluka Chemie AG
Fetal calf serum (FCS)	Omnilab
Fluo-3 AM	Fluka Chemie AG
Formaldehyde	Fluka Chemie AG
Formic acid	Sigma-Aldrich Chemie GmbH
N-Formyl-Met-Leu-Phe *BioChemika* (fMLP)	Sigma-Aldrich Chemie GmbH
Forskolin from *Coleus forskolii*, ≥98 % (HPLC)	Sigma-Aldrich Chemie GmbH
Geneticin® (G-418) (10131-019)	GIBCO® Invitrogen
Glass fibre filter type pcs G-7	Inotech Biologies

Glass fibre filter type GF/B	Whatman Switzerland GmbH
D-(+)-Glucose *BioChemika*	Fluka Chemie AG
L-Glutamine 200 mM (100X), liquid (25030-024)	GIBCO® Invitrogen
Glycerol 85 %	Hänseler AG
Guanosine 5'-O-diphosphate sodium salt, type I (GDP)	Sigma-Aldrich Chemie GmbH
Guanosine 5'-O-[gamma-thio]triphosphate tetralithium salt (GTPγS)	Fluka Chemie AG
Hanks' Balanced Salt Solution without calcium, magnesium, and phenol red (HBSS)	GIBCO® Invitrogen
n-Hexane (in house redistilled)	Fluka Chemie AG
Histamine (2-(4-imidazolyl)ethylamine)	Sigma-Aldrich Chemie GmbH
Hünig Base	Sigma-Aldrich Chemie GmbH
Hydrochloric acid ~6 M (HCl)	Sigma-Aldrich Chemie GmbH
Hydrogen peroxide 30 % (H_2O_2)	Hänseler AG
Hydroxylamine hydrochloride	Sigma-Aldrich Chemie GmbH
Imidazole	Fluka Chemie AG
Iscove's Modified Dulbecco's Medium	ATCC
Kaighn's Modification of Ham's F-12 Medium	ATCC
LDS Sample Buffer NuPAGE® (4X)	NOVEX® Invitrogen
Lecithin, commercial grade from soy bean	Hänseler AG
Lectin from *Phaseolus vulgaris BioChemika* (PHA)	Sigma-Aldrich Chemie GmbH
Lipopolysaccharide (LPS) from *E. coli*	Fluka Chemie AG
LymphoPrep™ (Axis-Shield, Oslo, Norway)	Omnilab
L-α-lysophosphatidylcholine (Egg, Chicken)	Avanti Polar Lipids Inc.
Magnesium acetate	Fluka Chemie AG
Magnesium chloride hexahydrate ($MgCl_2 \cdot 6\ H_2O$)	Fluka Chemie AG
Magnesium sulfate, anhydrous ($MgSO_4$)	Fluka Chemie AG
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7\ H_2O$)	Fluka Chemie AG
Methanol (MeOH)	Fluka Chemie AG
(R)-(-)-α-Methoxy-α-(trifluoromethyl)-phenylacetic acid chloride, 98 %	Sigma-Aldrich Chemie GmbH
Methyl t-butyl ether (MtBE)	Fluka Chemie AG
Methylene blue	Fluka Chemie AG
Methoxy arachidonyl fluorophosphonate (MAFP)	Tocris Biosciences
Milk powder, defatted	Migros-Genossenschafts-Bund
Miniumum Essential Medium Eagle	Sigma-Aldrich Chemie GmbH
Monobromobimane (MBrB)	Sigma-Aldrich Chemie GmbH
MOPS Buffer NuPAGE® (20X)	NOVEX® Invitrogen
NAN-190	Sigma-Aldrich Chemie GmbH

Nickel(II) chloride hexahydrate ($NiCl_2 \cdot 6\ H_2O$)	Carl Roth®
Ninhydrin	Fluka Chemie AG
Nitrocellulose Transfer Membrane, 0.2 µm	PerkinElmer
p-Nitrophenyl-β-D-glucopyranose	Sigma-Aldrich Chemie GmbH
Non-essential amino acids, MEM (100X)	Sigma-Aldrich Chemie GmbH
OptiPrep™ (Axis-Shield, Oslo, Norway)	Omnilab
Palmitic acid (hexadecanoid acid) ≥ 99 %	Sigma-Aldrich Chemie GmbH
Parthenolide	Sigma-Aldrich Chemie GmbH
PD 098059	Tocris Biosciences
Penicillin-Streptomycin stock solution (15140-122)	GIBCO® Invitrogen
Pentane (in house redistilled)	Fluka Chemie AG
Phloroglucinol	Sigma-Aldrich Chemie GmbH
Phorbol 12-myristate 13-acetate from euphorbiaceae *BioChemika* (PMA)	Fluka Chemie AG
Phosphate-Buffered Saline (PBS)	GIBCO® Invitrogen
L-α-phosphatidic acid (Soy) sodium salt	Avanti Polar Lipids Inc.
Phosphoric acid 85 % (H_3PO_4)	Fluka Chemie AG
Pluronic F-127	Sigma-Aldrich Chemie GmbH
Polyethylenimine (high molecular)	Sigma-Aldrich Chemie GmbH
Potassium chloride (KCl)	Hänseler AG
Potassium hydroxide (KOH)	Fluka Chemie AG
n-Propanol	Sigma-Aldrich Chemie GmbH
Propranolol hydrochloride	Sigma-Aldrich Chemie GmbH
Protease inhibitor cocktail (P8340)	Sigma-Aldrich Chemie GmbH
Protein Ladder SeeBlue® Plus 2 Pre-Stained Standard	NOVEX® Invitrogen
Pyridine	Sigma-Aldrich Chemie GmbH
all-*trans*-Retinoic acid (ATRA)	Sigma-Aldrich Chemie GmbH
Rhodamine 123	Fluka Chemie AG
RPMI-1640 Media	GIBCO® Invitrogen
Sample Reducing Agent NuPAGE® (10X)	NOVEX® Invitrogen
SB 202190	Tocris Biosciences
SB 203580	Tocris Biosciences
Scintillation cocktail Ultima Gold	PerkinElmer
Serotonin (5-hydroxytryptamine, 5-HT) as creatinin sulfate complex ($\cdot H_2O$)	Sigma-Aldrich Chemie GmbH
Silica gel Si60, 15–25, 15–40, and <63 µm	Merck KGa
Silica gel Si60, 40–65 µm	Fluka Chemie AG
Siliconizing Agent AquaSil™	Thermo Fischer Scientific

Silver nitrate ($AgNO_3$)	Fluka Chemie AG
Sodium butyrate	Sigma-Aldrich Chemie GmbH
Sodium carbonate (Na_2CO_3)	Fluka Chemie AG
Sodium chloride (NaCl)	Fluka Chemie AG
Sodium deoxycholate	Sigma-Aldrich Chemie GmbH
Sodium dithionite ($Na_2S_2O_4$)	Fluka Chemie AG
Sodium fluoride (NaF)	Carl Roth®
Sodium hydroxide (NaOH)	Fluka Chemie AG
Sodium lauryl sulfate (SDS)	Fluka Chemie AG
Sodium molybdate	Carl Roth®
Sodium nitrite	Sigma-Aldrich Chemie GmbH
Sodium orthovanadate	Sigma-Aldrich Chemie GmbH
Sodium potassium tartrate	Merck (Schweiz) AG
Sodium pyruvate 100 mM (100X)	Sigma-Aldrich Chemie GmbH
Sodium sulphate anhydrous (Na_2SO_4)	Sigma-Aldrich Chemie GmbH
SP600125	Tocris Biosciences
$sPLA_2$ from hog pancreas (600 U/mg, 1mg/ml, precipitated with ammonium sulphate)	Fluka Chemie AG
Sulphuric acid 98 % (H_2SO_4)	Fluka Chemie AG
Tetrachloro-*p*-benzoquinone	Sigma-Aldrich Chemie GmbH
Tetrachloromethane	Sigma-Aldrich Chemie GmbH
Tetrahydrofurane (THF)	Sigma-Aldrich Chemie GmbH
Thapsigargin	Alexis Biochem., Switzerland
TLC aluminium sheets, Silica gel $60F_{254}$, Silica gel 60	Merck KGaA
α-Tocopherol	Sigma-Aldrich Chemie GmbH
Toluene	Scharlau SA
p-Toluenesulfonic acid (p-TosOH)	Fluka Chemie AG
Transfer Buffer NuPAGE® (20X)	NOVEX® Invitrogen
Trichloro acetic acid	Sigma-Aldrich Chemie GmbH
Triethylamine (Et_3N)	Sigma-Aldrich Chemie GmbH
Tris(hydroxymethyl)aminomethane hydrochloride (TRIS-HCl)	Fluka Chemie AG
Tris(hydroxymethyl)-aminomethane (TRIS base)	Omnilab, Sigma-Aldrich Chemie GmbH
Triton® X-100	Fluka Chemie AG
Trypan Blue solution 0.4 %	Sigma-Aldrich Chemie GmbH
Trypsin, 0.05 % (1X) with 0.53 mM EDTA 4Na, liquid	GIBCO® Invitrogen
Tween-20	Fluka Chemie AG
Tween-80	Fluka Chemie AG
U0126	Tocris Biosciences
Umbelliferyl arachidonate	Cayman Chemicals

Vanillin	Fluka Chemie AG
Water LiChrosolv	Merck (Schweiz) AG
Wright-Giemsa Stain, Modified	Sigma-Aldrich Chemie GmbH
WST-1™ reagent	Roche Applied Science
X-ray photographic films, size A4	Kodak Society
Zymosan A from *Saccharomyces cerevisiae*	Sigma-Aldrich Chemie GmbH

3.2 Equipment

Equipment	*Comment*	*Supplier*
Centrifuge	Allegra™ centrifuges with rotors for tubes and plates, Optima™ TLX and Optima™ L-100 K ultracentrifuges with rotors type TLA 100.4 and 70 Ti	Beckman Coulter Inc.
Centrifuge, microliter	Heraeus® Biofuge® fresco, rotor type 3325	Heraeus Holding GmbH
Cytometer	Flow Cytometer FACScan™ with CellQuest™ V. 3.3, FCAP™ Array, and CBA analysis software, FACSCanto™ with FACSDiva™ software	Becton Dickinson
Dounce grinder	Dounce grinder 2 ml	Kontess Glass Co.
Electrophoresis	NuPAGE® 4-12 % Bis-Tris Gel 1.0 mm, 10 well, electrophoresis and blotting chambers	NOVEX® Invitrogen
Fluorescent image plate reader	GENios Pro (12903500039), V 2.26 10/03 with XFLUOR4GENIOSPRO Version V 4.53 software	Jasco GmbH
Fluorescence spectrophotometer	Varian Cary Eclipse Fluorescence Spectrophotometer with software version 1.1	Varian Inc.
HPLC columns, DAD detection	Nucleosil Symmetry® C_{18} column (3.5 µm particle size, 4.6 x 100 mm dimension, Part. No. WAT066220), Symmetry C_{18} column (5 µm particle size, 7.8 x 100 mm dimension, Part. No. 186000209), with precolumn cartridges	Waters Corp.
HPLC columns, fluorescence detection	Nucleodur Sphinx and Nucleodur Isis columns (each 4 µm particle size, 4.6 x 100 mm dimension) with precolumn cartridges	Macherey-Nagel GmbH & Co. KG
HPLC device	Elite LaChrome, HITACHI HPLC device with a diode array detector or a fluorescence detector	VWR International AG
IR spectrometer	IR spectrometer FT/IR-6200 and spectra manager software by Jasco	Jasco GmbH
Linomat	Linomat 2	CAMAG
Mass spectrometer	Alliance HT mass spectrometer with separation module 2795 coupled to a dual λ absorption detector 2487 and MassLynx V4.0 software	Waters Corp.

NMR spectrometer	Bruker 400 UltraShield and Bruker TOPSPIN 1.3 software	Bruker BioSpin
Rotary evaporator	Rotavapor®	Büchi Labortechnik AG
Scintillation counter	Beckman LS 6500 Multi-Purpose Scintillation Counter	Beckman Coulter Inc.
Shaker	Shaker KS10	Edmund Bühler
Sterile filters	For single-use, 0.2 μm	Millipore AG
TLC scanner	TLC Scanner 2	CAMAG
Transwell, 6 and 24 wells	Corning Costar® Transwell®-COL (6 and 24 well plates, 0.4 μm pore size)	Vitaris AG
Transwell, 24 wells	Biocoat® HTS Caco-2 Assay System (Cat.No. 354801)	Becton Dickinson
Ultrafiltration, 2 kDa	Vivaspin® 2 Hydrosat with 2 kDa MWCO	Sartorius Stedim Switzerland GmbH
Ultrafiltration, 50 kDa	Amicon Ultra-15, PLQK Ultracel-PL Membrane, 50 kDa MWCO	Millipore AG
Ultrasound bath	Waterfront membrane nebuliser, output: 24V, 1.2A, AC	Coop Gesellschaft
Vacutainer	Vacutainer LH 170 I.U., PrecisionGlide	Becton Dickinson
Voltohmmeter	Millicell ERS-1 system plus ‚chopstick' electrodes	Millipore AG
Voltohmmeter, electrode chambers	Endohm chambers for 24 mm and Costar Snapwell cup (for 6 well plates)	World Precision Instruments
Zetasizer®	Zetasizer® 3000HSA	Malverne Instruments Ltd

3.3 Kits for Molecular Biology

Kit	*Description*	*Supplier*
BrdU Flow Kit	FITC labelled BrdU Flow Kit	Becton Dickinson
Cell Signalling Master Buffer Kit		Becton Dickinson
Cytometric Bead Arrays	Human Inflammation Kit, Human Allergy Kit, Mouse Inflammation Kit	Becton Dickinson
ECL Plus™ Western Blotting Reagents	ECL Plus™ Western Blotting Detecting Reagents (Cat. No. RPN2132)	Amersham , GE Healthcare
FlexSets	IL-8, IL-1β, phospho ERK, phospho JNK, and phospho p38	Becton Dickinson
Human Soluble Master Buffer Kit		Becton Dickinson
Protein Quantification Assay	DC Protein Assay	BioRad Laboratories AG

3.4 Proteins

Protein	*Description*	*Supplier*
5-HT_{1A}R, protein	Membrane preparations from HEK-293 EBNA cells stably expressing human 5-HT_{1A} receptors	PerkinElmer
5-HT_3R, protein	Membrane preparations from HEK-293 EBNA cells stably expressing human 5-HT_3 receptors	PerkinElmer
CD28, antibody	Mitogenic mouse antibody against human CD28 (Cat. No. 348040)	Pharmingen, Becton Dickinson
CD3, antibody	Mitogenic mouse antibody against human CD3 ε-chain (isotype IgG1, κ, Cat. No. 555330) for Western blot, mitogenic mouse antibody against human CD3 Σ-chain (syn. for CD3 ζ-chain) (isotype IgG2a, κ, Cat. No. 555336) for live cell assays	Pharmingen, Becton Dickinson
$F(ab')_2$ fragment, PE conjugated	Sheep polyclonal $F(ab')_2$ fragment against full length mouse IgG, R-phycoerythrin (PE) conjugated (Cat. No. P8574)	Sigma-Aldrich Chemie GmbH
IgG, protein	Human IgG, reagent grade (purity ≥80 % by SDS-PAGE), as aqueous solution in 0.01 M phosphate buffered saline, pH 7.2, containing 15 mM sodium azide (Cat. No. I8640)	Sigma-Aldrich Chemie GmbH
IgG, protein, HRP labelled	Amersham ECL™ sheep anti-mouse IgG horse radish peroxidase labelled (Cat. No. NA931) and Amersham ECL™ donkey anti-rabbit IgG horse radish peroxidase labelled (Cat. No. NA934)	GE Healthcare
IL-1β, monoclonal antibody	Monoclonal mouse antibody against full-length human IL-1β protein (Cat. No. I3642)	Sigma-Aldrich Chemie GmbH
IL-1β, polyclonal antibody	Polyclonal mouse antibody against full-length human IL-1β protein (MaxPab, Cat. No. H00003553-B01P)	Abnova GmbH
IL-1β, protein	IL-1β standard from a CBA FlexSet	Pharmingen, Becton Dickinson
JNK1/2, antibody	rabbit polyclonal antibody against JNK1/2 (phospho T183 and Y185, ab4821)	Abcam
p38, antibody	Rabbit monoclonal antibody against phosphorylated p38 (phospho T180 and Y182, ab32557)	Abcam
Pro-IL-1β, protein	MaxPab lysate of a pro-IL-1β over-expressing cell line	Abnova GmbH
TNF-α, protein	Recombinant human TNF-α expressed in yeast (T0157), 10 µg/ml	Sigma-Aldrich Chemie GmbH

3.5 Plants and Extracts

Plant	*Description*	*Supplier*
AGP, purified	Purified arabinogalactan-proteins from *Physcomitrella patens* 'soluble AGP 4-2' [307] and *Raphanus sativus* 'mature root AGP' and 'AGP-1'	Prof. E. A. Nothnagel, Dept. of Botany and Plant Sciences, University of California, Riverside, CA, USA
Agropyron repens, plant	*Graminis rhizoma PhH 5*	Hänseler AG
Alpinia sp., plant	*A.* cf. *officinarum* rhizome, integrated production	Migros Genossenschaft
Apium graveolens, plant	*A. graveolens* var. *rapaceum* root, integrated production	Migros Genossenschaft
Armoracia rusticana, plant	*A. rusticana* root, integrated production	Migros Genossenschaft
Beta vulgaris, plant	*B. vulgaris* subsp. *vulgaris* var. *conditiva* root, integrated production	Migros Genossenschaft
Brassica oleraceae, plant	*B. oleraceae* convar. *capitata* var. *sabauda* leaves, integrated production	Migros Genossenschaft
Cucurbita pepo, plant	*C. pepo* ssp. *pepo* convar. *giromontiina* fruit, integrated production	Migros Genossenschaft
Daucus carota, plant	*D. carota* subsp. *sativus* root, integrated production	Migros Genossenschaft
Dryopteris filix-mas, plant	*D. filix-mas* rhizome	Hänseler AG
Echinacea purpurea, root	Organic *E. purpurea* root	Bioforce AG
Harpagophytum procumbens, extract	Extracts of organic *H. procumbens*	Bioforce AG
Equisetum arvense, plant	*E. arvense* herb	Hänseler AG
Foeniculum vulgare, plant	*F. vulgare* var. *vulgare* fruit	Hänseler AG
Ononis spinosa, plant	*O. spinosa* root	Hänseler AG
Raphanus sativus, plant	*R. sativus* subsp. *niger* var. *albus* root, integrated production	Migros Genossenschaft
Salix sp., extract	Extract of *Salix* cortex (Extr. Salicis e cort. Spir. Sicc., Ch. No. 0400750).	Bionorica SE
Thymus vulgaris, plant	*T. vulgaris* leaves	Hänseler AG
Zingiber officinale, essential oil	Essential oil from commercial grade ginger rhizome, water vapour distillate	Farfalla Essentials AG

Zingiber officinale, extracts, CO_2	Extract of *Z. officinale* rhizome (ginger Hot Flavor™ CO2-to extract, Type No. 014.088, Ch. No. 120507 and ginger CO2-to extract, Type No. 014.002, Ch. No. 440101). Carbon dioxide extraction according to Manninen et al. [308].	FLAVEX Naturextrakte
Zingiber officinale, extracts, ethanolic	Extracts of organic *Z. officinale* rhizomes (India, strain 'Rio de Jenairo', Ch. No. V61101, fresh rhizome with 85 % moisture content, 75 % ethanol, DER 1:30, dry matter 0.418 % and Ch. No. V61501, dried rhizome with 13.2 % moisture content, 90 % ethanol, DER 1:10, dry matter 0.427 %)	Bioforce AG
Zingiber officinale, plant	Organic *Z. officinale* fresh rhizomes (food grade from China via HPW Marketing and from India strain 'Himachal' via Erboristi Lendi, Curio, Switzerland)	Bioforce AG

3.6 Self-made Reagents

3.6.1 TLC Detection Reagents

Anisaldehyde Reagent R [309]:

Spray with a solution containing 0.5 ml p-anisaldehyde, 5 ml H_2SO_4 98 %, and 10 ml glacial acetic acid in MeOH at 100 ml and gently heat to ~110 °C.

Basic polyethylenimine, adapted from [310]:

Spray with 1 % NaOH and 1 % polyethylenimine (high molecular) in MeOH.
NaOH was used to hydrolyse the umbelliferyl arachidonate ester whilst polyethylenimine prolonged and enhanced fluorescence.
Dry <80 °C and detect under UV at 366 nm.

Cerium-ammonium-molybdate (modified Hanessian's Stain):

Spray with a solution of 5 g of ammonium heptamolybdate tetrahydrate, 0.2 g of cerium (IV) sulfate, 10 ml H_2SO_4 98 %, and 90 ml water and heat to ~150 °C.

Chloranil/NH_3 and Chloranil/H_2SO_4, adapted from [310]:

Spray with 1 % tetrachloro-*p*-benzoquinone in toluene and let dry at RT.
Either put in an ammonia vapour chamber or spray with 1 % H_2SO_4 in EtOH.

Diphenylamine/Aniline [310, 311]:

Prepare a solution of 2 ml aniline, 2 g diphenylamine, and 10 ml H_3PO_4 85 % in 100 ml acetone and store tightly sealed at 4 °C in the dark. After spraying, Heat the plate in an oven at 85 °C for 10'.

Molybdenum Blue, modification of [312]:

Prepare a solution of 742 mg (6 mmol) ammonium heptamolybdate tetrahydrate, 243.6 mg (14 mmol) $Na_2S_2O_4$, 2 ml H_2SO_4 98 %, and 100 ml 20 % EtOH and keep it at 4 °C for at least 1 day before use. Store the solution at 4°C up to one month.
Let the plate completely dry at 50-60 °C after spraying, then slowly heat to ~110-120 °C and if desired char at 150 °C.

Ninhydrin, modification of [313]:

Spray with 0.2 % ninhydrin in acetone and gently heat to 110 °C.

Tollens Reagent [311]:

Prepare a solution of 2 % $AgNO_3$ and 1 % NaOH and add enough NH_3 30 % to readily dissolve the precipitate and store at 4°C in the dark. Gently heat to ~70 °C after spraying.

Vanillin/H_2SO_4 [310]:

Spray first with 1 % vanillin in EtOH, than with 15 % H_2SO_4 in EtOH, and gently heat plate to ~110 °C.

3.6.2 Phosphatase Inhibitor Cocktail

The final concentration of the single compounds of the used phosphatase inhibitor cocktail were as follows: 4 mM sodium potassium tartrate, 2 mM imidazole, 1.15 mM sodium molybdate, 1 mM sodium fluoride, and 1 mM sodium orthovanadate[1)].
Each compound was prepared as a 500 X solution in water and subsequently combined to equal volumes resulting in a pH ~9.7. This 100 X stock solution was frozen at -20 °C in 10 µl aliquots. Samples which turned deep yellow were discarded.
Where desired, the pH was adjusted with HCl to pH 8 (50 µl stock solution, 25 µl 0.1 M HCl, 1 ml BD Denaturation Buffer, and 3.925 ml water).

In some cases, the stock solution was diluted directly before use with 23.3 µl water to obtain a 30 X stock.

[1)] Sodium orthovanadate solution was prepared according the abcam handout 'Buffers and Stock Solutions' [314] as follows:
- A 500 mM solution was prepared and the pH set to 9.0 with HCl
- The solution was boiled until it was colourless and cooled to RT
- The pH was set again to 9.0 with HCl
- The solution was again boiled until it was colourless and cooled to RT
- This was repeated until the solution stayed at pH 9.0 when heating and cooling
- The volume was adjusted

3.6.3 Modified RIPA Buffer

The modified RIPA buffer was prepared as aqueous stock solution containing 55.55 mM TRIS base, 111.11 mM NaCl, 11.11 % [$^{m}/_{m}$] glycerol, 1.11 % [$^{m}/_{m}$] Triton® X-100, 1.11 % [$^{m}/_{m}$] 2-mercaptoethanol, 0.55 % [$^{m}/_{m}$] sodium deoxycholate, 0.11 % [$^{m}/_{m}$] SDS, 5.55 mM EDTA, 5.55 mM EGTA, adjusted to pH 8 with HCl.
Immediately before use, 27 volume parts modified RIPA buffer were supplemented with 2 vol. parts Protease Inhibitor Cocktail and 1 vol. part Phosphatase Inhibitor Cocktail (30 X).

3.7 Isolation and Purification

3.7.1 Extractions

Oleoresin extraction from *Zingiber officinale* rhizomes:

1: 1 kg fresh ginger rhizome was cleaned and cut into small pieces (~5 mm), frozen at -80 °C and lyophilised.

2: The lyophilisate was crushed, mixed with 2 l acetone, mashed in an Ultra-Turrax® and macerated over night.

3: The extract was filtered and extraction was redone. The extracts were combined and dried on a Rotavapor®.

4: The residue was exhaustively extracted (~5 times) with each time 2 l diethyl ether (Et_2O), the extracts were dried on a Rotavapor® and combined with the acetone extract.

5: The extract was dissolved in Et_2O and shaken out against one part water. The water phase was completely re-extracted with Et_2O. The anorganic phase was dried over anhydrous Na_2SO_4 and dried on a Rotavapor®.

6: The extracted oleoresin was pre-purified over a small plug of silica using an eluent of n-hexane/ethyl acetate (EtOAc) (1:1) to be subjected to flash liquid chromatography (flash LC) (see chapter 3.7.3).

AGP rich plant extracts for *in vitro* assays were prepared as follows:

1: About 50 g of fresh plant material were washed in deionised water and grated or about 10 g of dried plant material were powdered in a hammer mill.

2: 50 ml acetone was added and macerated for at least 30 min and filtered trough a sinter glass filter Nor. 4 and washed with acetone.

3: Residue was re-suspended in 50 ml Et_2O and macerated for at least 30 min and filtered again.

4: Step 3 was repeated once and residue washed with Et_2O.

5: Residue was re-suspended in sufficient distilled water (10-40 ml) and macerated over night and then filtered over sinter glass filter No. 4 and washed with water.

6: Filtrate was filtered trough a Millipore filter (0.22 μm pore size).

7: The solution was lyophilised.

8: Extracts were tested for lack of LPS-contamination at Cambrex Corporation, UK.

Daucus carota AGPs for mouse experiments were isolated as described above but lyophilised powdered fresh material was used and instead of filtering in point 5 the extract was centrifuged for 10' at 1'000 g and filtered through two strengthened paper filters and lyophilised again. Two times 500 mg extract were mixed with 50 mg Yariv's reagent dissolved in 50 ml 0.15 M NaCl solution and kept at 4 °C over night and centrifuged for 10' at 2'000 g. Precipitate was dissolved in a minimal amount of DMSO. Three parts acetone and 0.08 parts 1 % sodium chloride solution were added and stored at 4 °C over night. The precipitate was spun down for 10' at 700 g. The pellet was dissolved in DMSO and precipitated again. The precipitate was dissolved in 8 ml water and ultrafiltrated trough Vivaspin® 2 Hydrosat with 2'000 MWCO (at 3'000 g for 30 min) and resuspended twice with 2 ml water and ultrafiltrated again and finally lyophilised. AGPs were tested LPS-free at Cambrex Corporation, UK.

AGP deprived extracts and purified AGPs for whole blood assays were prepared from aqueous extracts of *Daucus carota*, *Dryopteris filix-mas*, *Equisetum arvense*, *Thymus vulgaris*, and *Zingiber officinale.* Each 1 mg/ml aqueous extract solution was mixed with 1 mg/ml aqueous Yariv's reagent and stored at 4 °C over night and centrifuged at 10'000 g for 30 min. The supernatant was removed and 1 % NaCl was added and kept at 4 °C over night and centrifuged again. The combined precipitates were dissolved in 200 µl water and 30 µl of a 5 % $Na_2S_2O_4$ solution was added and heated to 56 °C until discoloration. Solutions were shock-frozen and lyophilised and then solved in a 30 µl 1 % sodium chloride solution. 330 µl DMSO were added and finally 990 µl acetone and stored at 4 °C over night [289]. The precipitate was spun down for 10' at 700 g, precipitation was redone, and the final precipitates were lyophilised. The AGP deprived extracts and the isolated AGPs were diluted with 1 ml PBS and frozen at -18 °C.

3.7.2 TLC

Ginger TLC

Several solvent systems for thin layer chromatography (TLC) were analized for the separation of ginger extracts and gingerol-type compounds:

- According the European pharmacopoeia: n-hexane/Et_2O (3:2)
- Gingerols/shogaols: n-hexane/Et_2O/EtOAc (2:2:1)
- Gingerols/shogaols: n-hexane/Et_2O/EtOAc/AcOH (40:40:20:0.5)
- Gingerols/shogaols: n-hexane/Et_2O/AcOH (20:20:1)

Detection: UV 254 and 366 nm, vanillin/H_2SO_4, anisaldehyde reagent R, cerium-ammonium-molybdate, Tollens reagent, chloranil/NH_3, or chloranil/H_2SO_4 spray reagents.

Essential oils and terpenes were separated with toluene/ethyl acetate (93:7) [315].
Detection: UV 254 and 366 nm and vanillin/H_2SO_4.

The following solvent systems were used for hydrophilic compounds including polysaccharides and proteins:
- Hydrophilic small molecules: $CHCl_3$/MeOH/water/AcOH (85:13:1.5:0.5)
- Polysaccharides: n-butanol/MeOH/water (4:3:3)
- Proteins: $CHCl_3$/MeOH/water/AcOH (70:40:9:1)
- Proteins: n-propanol/EtOAc/water/ammonia 30 % (60:40:15:10)

Detection: UV 254 and 366 nm, vanillin/H_2SO_4, ninhydrin, and diphenylamine/aniline spray reagents.

Lipid TLC

Crude lipid preparations or second opinion separations were done with a solvent system of $CHCl_3$/MeOH/AcOH/water (70:30:8:4) and detection was done with molybdenum blue spray and charring.

Phospholipid TLC

Crude phospholipid containing preparations were separated by a TLC method described by Weerheim et al. [316] with the modification of using normal TLC aluminium sheets. The solvent system was $CHCl_3$/MeOH/EtOH/EtOAc/ acetone/isopropanol/water/AcOH (30:28:16:6:6:6:6:2) and detection was done with molybdenum blue spray and charring.
Enzymatic reactions using purified phosphatidylcholine were separated by a TLC solvent system of $CHCl_3$/MeOH/AcOH/water (17.5:7.5:2:1) [317, 318]. Detection was done with molybdenum blue spray and charring.

2-Dimensional Phospholipid TLC

2-D TLC was done strictly according to [319]. Sample were applied in one corner and ran with $CHCl_3$/MeOH/formic acid/water (60:30:7:3), the plate was dried under nitrogen and ran in a 90° angel with $CHCl_3$/MeOH/ammonia 30 %/water (50:40:8:2), dried again and ran in the opposite direction with Et_2O.

Umbelliferon TLC

Enzymatic reactions using umbelliferyl arachidonate were separated by a TLC solvent system of $CHCl_3$/Et_2O/toluene/n-hexane/AcOH (20:10:10:10:1), alternative DCM/Et_2O/AcOH (20:20:1) could be used. Detection was done with basic polyethylenimine and UV 366 nm.

Bimane Derivatives

Bimane reaction mixtures from enzymatic thio-PC hydrolysis were separated by TLC during assay development using a solvent system of $CHCl_3$/EtOAc/acetone/MeOH/water/Hünig base (35:30:20:20:4.5:2.5). Detection was done by molybdenum blue spray and charring for unreacted phospholipids and for bimane adducts under UV at 366 nm. This TLC technique gives a beta front at R_f-value 0.5 with phospholipids below and bimane derivatives above. R_f-values for phospholipids and thio-PC were 0.4 to 0.5, for lyso-derivatives <0.2, bimane adduct of lyso-thio-PC 0.45, bimane derivatives 0.6 to 0.8 and pure bimane at the front.

Bimane reaction mixtures from chemical thio-PC hydrolysis were separated by TLC during assay development using a solvent system of $CHCl_3$/MeOH/water/n-butanol/AcOH (4:4:1.5:1:1). Detection was done by molybdenum blue spray and charring for unreacted phospholipids and for bimane adducts under UV at 366 nm. R_f-values for phospholipids and thio-PC were 0.7 to 0.8, for lyso-derivatives ~0.5, bimane adduct of lyso-thio-PC 0.66, bimane adducts of DTT > 0.8 and pure bimane at the front. Bands were scratched away, extracted and determined in ESI-MS.

3.7.3 Flash LC

Flash liquid chromatography was used as first isolation step for gingerol-enriched fractions and 5-HT_{1A}R ligands.

The aforementioned extracted oleoresin was separated with flash LC over 500 g silica gel Si60 (40–65 μm) on a column with 8 cm diameter and pentane/Et_2O (1:1) as eluent. Fractions were monitored by TLC with a solvent mixture of n-hexane/Et_2O/acetic acid (AcOH) (20:20:1). Equal fractions were combined resulting in 16 final fractions (Fig. 9) of which fraction 11 (155-165) was used for the synthesis of Mosher's ester.

The ginger Hot Flavor™ extract was used as starting material for the isolation of serotonin receptor ligands by separation with flash LC as follows.

A first isolation with 500 mg resulted in fractions too small for the isolation of pure compounds. The extract was separated over ~40 cm^3 silica gel (40–65 µm) in a two cm flash-chromatography column with pentane/Et_2O 1:1 as eluent giving 40 fractions where similar ones were combined into 10 fractions which were tested in the radioligand displacement assay. Fraction 7 was further separated on HPLC.

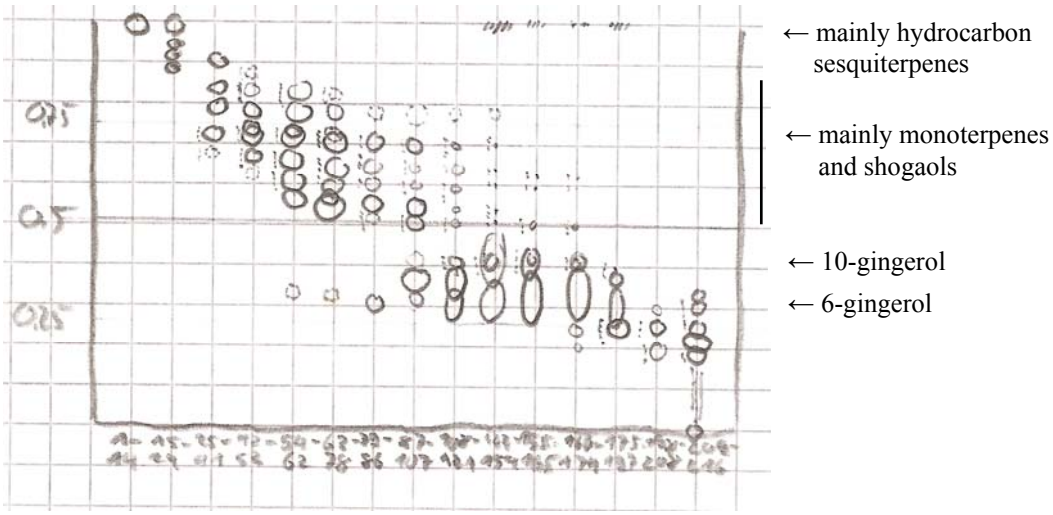


Figure 9: Thin layer chromatography profile.

TLC profile of the 15 fractions obtained by flash chromatography of a crude extract from fresh ginger rhizome with pentane/Et_2O (1:1) as eluent. The TLC solvent system was n-hexane/Et_2O/AcOH (20:20:1) and TLC plates were detected with vanillin/H_2SO_4 spray reagent.

For the second and successful isolation 18.9 g extract were separated over ~800 cm^3 silica gel (40–65 µm) in an eight cm column with pentane/Et_2O 1:1 as eluent resulting in 110 fractions. The column was washed with EtOAc (one fraction) and finally with acetone/EtOH 1:1 and MeOH/water 2:1 (another fraction). All fractions were analyzed by TLC and HPLC. Similar fractions were combined resulting in 14 fractions (F1 – F14) of which F2-F9 were tested in the radioligand displacement assay.

Fraction 3 (4.5171 g) was further separated by flash LC over ~100 cm^3 silica gel (15-25 µm) in a 6 cm column with cyclohexane/dioxane 7:1 as eluent resulting in 76 fractions. Washout with a 1:1 mixture gave another 5 fractions. All fractions were analyzed by TLC

and HPLC. Similar fractions were combined resulting in 11 fractions (F3.1 – F3.11) and tested in the radioligand assay.

Fraction 3.6 (~1.67 g) was separated over 100 cm^3 silica gel (15-40 μm) in a 4 cm column. The eluent was pentane/methyl t-butyl ether/DCM/EtOAc 15:1:1:0.5 resulting in 51 fractions. The column was washed with EtOAc (5 fractions) and isopropanol (5 fractions). Similar fractions were combined due to TLC chromatograms resulting in 8 fractions (F3.6.1 – F3.6.8).

Fraction 3.7 (~310 mg) was separated over 50 cm^3 silica gel (15-40 μm) in a 3 cm column. The eluent consisted on pentane/DCM/methyl t-butyl ether/ethyl formate 10:2.5:1:0.5 resulting in 25 fractions. The column was rinsed with a gradient containing stepwise less pentane resulting in another 13 fractions. Finally the column was washed with acetone giving 6 fractions. Fractions were combined due to similar TLC chromatograms resulting in 10 fractions (F3.7.1 – F3.7.10).

Fraction 3.8 (~127 mg) was separated over 12 cm^3 silica gel (<63 μm) in a 1 cm column. As eluent pentane/DCM/methyl t-butyl ether/ethyl formate/THF 10:2:1:0.25:0.25 was used resulting in 20 fractions, followed by an eluent of methyl t-butyl ether/ethyl formate/THF 1:0.25:0.25 (2 fractions), and one wash-out fraction using acetone. TLC chromatograms were done to combine similar fractions resulting in 8 different ones (F3.8.1 – F3.8.8).

Fraction 3.9 (~50 mg) was separated over 10 cm^3 silica gel (<63 μm) in a 1 cm column. The eluent was cyclohexane/Et_2O/THF 10:2:1 resulting in 20 fractions, followed by 1 fraction with Et_2O/THF 2:1 and 2 fractions with acetone. Similar fractions were combined due to TLC chromatograms resulting in 8 fractions (F3.9.1 –F3.9.8).

Fraction 7 (218.4 mg) was further separated by chromatography over ~70 cm^3 silica gel (15-25 μm) in a 4 cm column with pentane/dioxane 6:1 as eluent resulting in 59 fractions. Washout with a 1:1 mixture gave another fraction. All fractions were analyzed by TLC and HPLC and similar ones were combined resulting in 19 fractions (F7.1 – F7.19) of which all but the mixed fractions F7.7, 7.9, 7.11, 7.13, and 7.15 were tested in the radioligand assay. NMR-analysis of fractions F7.3, 7.8, 7.12, and 7.14 showed that they were mixtures even tough HPLC-chromatograms were relatively clean. Fraction 7.16 consisted of mainly 10-gingerol which was used for the synthesis of 10-shogaol.

3.7.4 HPLC

The following HPLC methods were used to separate and purify ginger constituents.

For the separation of the fraction number 7 of the first flash LC separation a gradient on an analytical column was used: w/a 60:40 for 2', gradient over 7' to w/a 10:90, isocratic for 1', gradient to w/a 60:40 during 1' and equilibration for 1' with a flow rate of 1 ml/min. Eight fractions were collected contained between 20 to 80 µg and were tested in the radioligand displacement assay but no further used.

The following high-performance liquid chromatography methods were used to isolate 5-HT_{1A}R ligands using gradients with water/acetonitrile (w/a) or water/acetonitrile/MeOH (w/a/m).

Gradient A (analyt. and prep. used): w/a 60:40 for 2', gradient over 7' to w/a 10:90, isocratic for 1', gradient to w/a 60:40 during 1' and equilibration for 1'. R_f values: 6-G 2', 8-G 4', 6-S 5-5.5', 8-S 6-7', and 10-G 7-8', 10-S 10-11'.

Gradient B (only prep. used at a flow rate of 2.5 ml/min): w/a 50:50 for 2', gradient over 7' to w/a 5:95, gradient to w/a/m 5:85:10 for 5', gradient to w/a 50:50 for 2' and equilibration for 1'. R_f values: 10-G 9', 10-S 11'

Gradient C (analyt. used at a flow rate of 1 ml/min): w/a 60:40 for 2', gradient over 18' to w/a 5:95, isocratic for 1', gradient to w/a 60:40 for 2' and equilibration for 1'.

Fractions F3.6 – F3.9 were separated by prep. HPLC with gradient B and subfractions thereof with gradient A.

Comparison of HPLC-chromatograms and data from radioligand assays of the subfractions of F3.7 – F3.9 were used to determine the subfractions to be further separated. The following fractions were promising because of good activity, enough material and only 1 to 3 main constituents. Individual gradients were used for each fraction: F3.6.6 (two times: 3.41 and 5.0 mg), F3.7.2 (4.7 mg), F3.7.3 (two times: 2.06 and 3.5 mg), F3.8.3 (2.30 mg), F3.9.3 (2.0 mg) and F3.9.5 (2.11 mg). Pure constituents were purified again by HPLC, analysed with MS and 2D-NMR and identical ones were combined.

Fraction F7.3 (4.12 mg) was separated by prep. HPLC with the gradient A resulting in 22 fractions and one waste fraction (F7.3.1 - F7.3.22 and F7.3.W). Acetonitrile was deprived on a Rotavapor®, 40 °C and reduced pressure and water was removed by lyophilisation.

Fractions F7.8 (22.64 mg), F7.12 (9.78 mg), F7.14 (18.55 mg) and F7.16 consisted each of one main peak on TLC and were therefore purified by HPLC with individual methods and treated equal to F7.3.12 which gave for F7.8: 5 fractions, for F7.12: 8 fractions and for

F7.14: 6 fractions. The main constituents as well as the subfractions containing minor compounds were again tested in the radioligand assay.
All active fractions of F7.3 contained one main constituent of each less than 0.2 mg and could not be characterized. F7.12 contained two active fractions which were mixtures. Further separation gave fractions with less than 0.2 mg and could therefore not be analysed.

3.8 Cell Culture and Cellular Assays

3.8.1 Whole Blood and Primary Cells

Human whole blood was drawn by venipuncture from healthy human volunteers of both sexes at the age of 20 to 40 using the Vacutainer system by BD to be used mainly for whole blood assays and the isolation of primary monocytes. Isolation of fresh peripheral blood was conducted in accordance with the guidelines of the World Medical Association's Declaration of Helsinki and approved by ETH Zurich.
Fresh buffy coats for the isolation of leukocytes were provided by the local blood donation centre and were from donors of either sex between 20 and 40 years.
Human monocytes and lymphocytes were isolated from freshly drawn blood or buffy coats using the density gradients OptiPrep™ according the Axis-Shield Application Sheets No. C9 and LymphoPrep™, respectively, strictly according to the manufacturer's recommendations and were further purified by adhesion to plastic surfaces for 15-30 min. Adherent monocytes were detached with ice cold HBSS (w/o Ca^{2+}/Mg^{2+}) for 10-15 min. Leukocytes were either cultured in RPMI-1640 medium containing 2 mM glutamate, 2 µg/ml Amphotericin B, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10 % fetal calf serum at 37 °C, 5 % CO_2 and humidified atmosphere or frozen in culture medium containing 10 % DMSO and stored at -80 °C.
BALB/c wild type mice and $MyD88^{-/-}$ and $CD14^{-/-}$ knock-outs on a BALB/c background were a gift from Prof. Dr. Urs Eriksson, Institute of Physiology, University of Zurich. TLR4 kinase dead mice ($TLR4^{d/d}$, C3H/HeJ on a BALB/c background) were a gift from Prof. Dr. Manfred Kopf, Institute of Integrative Biology, ETH Zurich.

3.8.2 Cell Lines

Caco-2 (HTB-37™): Caco-2 cells were from ATCC, Rockville, MD. Culture medium was according to the ATCC recommendations and as described by Rothen-Rutishauser et al. [320] with the addition of 2 µg/ml Amphotericin B: Minimum Essential Medium Eagle

supplemented with 2 mM glutamate, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 2 µg/ml Amphotericin B, 100 units/ml penicillin, 100 µg/ml streptomycin, and 20 % fetal calf serum (FCS) at 37 °C, 5 % CO_2 and humidified atmosphere. Cells were subcultured when they reached around 80 % confluence (~$8 \cdot 10^4$ to $1 \cdot 10^5$ cells/cm^2) and seeded at $1 \cdot 10^4$ cells/cm^2 for further cultivation/propagation. FCS was chosen from four batches (Omnilab, Gibco) by optimal proliferation and minimal dome formation.
CHO-K1 clones: CHO-K1 cells (CCL-61™) and CHO-K1 cells stably expressing the human TRPV1 (CHO20-5-10) were a gift from Zoltan Sandor, University of Debrecen, Hungary. The CHO-K1 clone stably expressing the human CB2 receptor was from Prof. Dr. Jürg Gertsch [321]. The cell lines were cultured strictly according the ATCC recommendations for CHO-K1 cells.

HeLa clone HA-6: The HeLa clone HA-6 [322] stably expressing the human 5-HT_{1A}R was a gift from Novartis, Switzerland. Cells were cultured strictly according the aforementioned publication: Dulbecco's Modified Eagle's Medium containing 2 mM glutamate, supplemented with 400 µg/ml geneticin®, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10 % fetal calf serum (FCS) at 37 °C, 5 % CO_2 and humidified atmosphere. Cells were subcultured when they reached around 80 % confluence.

HL-60 (CCL-240™): The HL-60 cell line was from ATCC, Rockwell, MD. Cells were cultured strictly according the ATCC recommendations using ATCC culture medium.

KB (CCL-17™): The KB cell line was from ATCC, Rockwell, MD. Cells were cultured according the ATCC recommendations in Minimum Essential Medium Eagle supplemented with 2 mM glutamate, 2 µg/ml Amphotericin B, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10 % fetal calf serum (FCS) at 37 °C, 5 % CO_2 and humidified atmosphere.

Jurkat, clone E6-1 (TIB-152™): The Jurkat clone E6-1 was from ATCC, Rockville, MD. Culture medium was standard RPMI-1640 medium supplemented with 2 mM glutamate, 2 µg/ml Amphotericin B, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10 % fetal calf serum at 37 °C, 5 % CO_2 and humidified atmosphere.

MCF7 (HTB-22™): The MCF7 cell line was from ATCC, Rockwell, MD. Cells were cultured according the ATCC recommendations but without insulin in Minimum Essential Medium Eagle supplemented with 2 mM glutamate, 2 μg/ml Amphotericin B, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 % fetal calf serum (FCS) at 37 °C, 5 % CO_2 and humidified atmosphere.

U-937 (CRL-1593.2™): The U-937 cells [323] were from ATCC, Rockville, MD and were cultured according the ATCC recommendations: RPMI-1640 medium containing 4.5 g/l glucose, 1.5 g/l sodium bicarbonate, 2 mM glutamate, 1 mM sodium pyruvate, supplemented with 2 μg/ml Amphotericin B, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 % fetal calf serum (FCS) at 37 °C, 5 % CO_2 and humidified atmosphere.

General procedures:

Sub-cultivation of adherent cell lines was performed as follows: medium was removed and cells were washed with ice cold HBSS (w/o Ca^{2+}) and strongly adherent cells were additionally incubated for 30' at 4 °C. Cells in T75 tissue culture flasks were rinsed for one minute with 3 ml trypsin/EDTA solution (proportionally less for T25) and incubated with 2 ml fresh trypsin/EDTA solution at RT for 5 to 10 min. Strongly adherent cells were incubated at 37 °C. When the cells started to detach trypsin was removed and cells were incubated again for around 5 min until ~80 % could be detached by gently tapping. Culture medium was added and eventual cell aggregates were disintegrated by gentle pipetting. Cells were counted in a Neubauer Improved haemocytometer and adjusted to the desired cell density.

For freeze storage cells were brought to RT and distributed at desired amounts into freeze vials. 10 % DMSO was added dropwise during at least 10 min with regular gentle vial inversion. Vials were sealed and cooled down to 4 °C and frozen in a closed Styrofoam box at -80 °C. For long-term storage cells were transferred into liquid nitrogen the day after.

Frozen cells were rapidly thawed and diluted 1:9 in culture medium and spun down at 200 g for 3 min. The pellet was suspended in pre-equilibrated 37 °C warm cultured medium to achieve an appropriate cell density.

3.8.3 Human Whole Blood Assay

Human whole blood was immediately pipetted in a 96-well plate, 200 µl each well. Vehicle controls, extracts, or pure compounds were added and incubated for 30 min at 37 °C, 5 % CO_2 in humidified atmosphere. Then, vehicle controls or stimuli were added and cells were incubation of 18 h.

The plates were centrifuged at 500g for 5 min and an appropriate amount of supernatant serum was taken for immediate analysis with CBA according to the manufacturer's recommendations but with minor changes as described below (Human Allergy Mediators Kit is off the market; the handling was identical with the newly formulated Human Inflammation Kit), measured with FACScan, and evaluated with the CBA software.

The assay procedure for the ancient formulations was as follows:

1: Capture Beads were vortex, mixed and centrifuged at 200*g* for 5 minutes. Supernatant was replaced with serum enhancement buffer and incubated for 30 min at RT.
2: 10 µl mixed Capture Beads were added to all assay tubes.
3: 10 µl of each sample or the included cytokine standard dilution, prepared according the manufactures manual, were added to the tubes.
4: The tubes were incubated for 1.5 h at RT and protected from light.
5: 200 µl of Wash Buffer were added to each assay tube and centrifuge at 200*g* for 5 min.
6: The upper 200 µl of the supernatant were carefully aspirated and discarded.
7: 10 µl of the PE Detection Reagent was added to all assay tubes.
8: The tubes were incubated for 1.5 h at RT and protected from light.
9: 200 µl of Wash Buffer were added to each assay tube and centrifuged at 200*g* for 5 min.
10: The supernatant was carefully aspirated and discarded from each assay tube.
11: 60 µl of Wash Buffer were added to each assay tube and the bead pellet was resuspended.
12: Samples were measure at low flow on a FACScan and evaluated with CBA analysis software.

The assay procedure for new formulations was the following:

1: Identical to steps 1-3 in above mentioned protocol
2: 10 µl of the PE Detection Reagent was added to all assay tubes.

3: The assay tubes were incubated for 3 hours at RT and protected from light.

4: Identical to steps 9-12 in above mentioned protocol

In a first step, several compounds were tested for their ability to induce cytokine expression with the aim of using them as inflammation model. The imposed requirements were that the stimuli or combinations thereof had to i) induce as many cytokines as possible, ii) cause a strong induction (100-1'000 fold increase compared to constitutive levels), and iii) at best, involve known distinct inflammation-related pathways or receptors (e.g. TLR4, TCR).
In a second step, several compounds known to interfere with inflammation-related pathways, enzymes, or receptors were tested in stimulated whole blood to i) validate the whole blood assay and as prove of concept for its suitability as inflammation model, ii) use them as positive controls, and iii) get 'inhibition/modulation' patterns. The latter might prove helpful as comparison to the modulation patterns of plant preparations. Notably, some of the used stimuli showed modulating behaviour too (e.g. forskolin or all-*trans*-retinoic acid).

3.8.4 Mouse Whole Blood Assay

Mice were euthanized in a carbon dioxide atmosphere. Blood was taken from the beating heart, collected in heparin (8 I.E./ml) containing Eppendorf tubes, and immediately diluted 1:1 with Hanks Balanced Salt Solution (HBSS) at 37 °C. 100 µl aliquots were dispensed in 96-well plates, treated with vehicle control, LPS (100 ng/ml), zymosan A (50 µg/ml), and *D. carota* AGPs (50 µg/ml), respectively, and incubated for 18 hours at 37 °C, 5 % CO_2 in humidified atmosphere. Thereafter, the plates were centrifuged at 500 g for 5 min and 10 µl supernatant used for cytokine detection with the CBA Mouse Inflammation Kit according to the manufacturer's recommendations but with minor modifications as described in chapter 3.8.3 for the new formulations and with 2 h instead of 3 h incubation in point 3.

3.8.5 *In Vivo* Mouse Experiments

D. carota AGP, LPS, zymosan A, and carrageenan were tested in mice for paw edema formation and inflammatory marker induction by Dr. Juan Manuel Viveros-Paredes, Instituto Mexicano del Seguro Social, Mexico.

Paw edema were induced by intra-epidermal injection of 20 µl saline or a 0.2 % saline solution of carrageenan, *D. carota* AGP, or zymosan A. Paw size was measured at time zero and after 30, 60, and 90 min.

Levels of plasma nitric oxide, IL-1β, and TNF-α were measured after intra-peritoneal injection of 20 µl saline or a saline solution of LPS (250 µg / 100 g), *D. carota* AGP (1 mg / 100 g), or zymosan A (1 mg / 100 g) as described elsewhere [324].

3.8.6 Absorption Model

Cells were gathered at around 80 % confluence (~$8 \cdot 10^4$ to $1 \cdot 10^5$ cells/cm^2), seeded on Transwell-COL membrane inserts (6 well plate) at a density of $1 \cdot 10^5$ cells/ml.

They were cultured for about three weeks to reach a trans-epithelial electrical resistance (TEER) exceeding 400 Ω/cm^2 determined at 37 °C using a Millicell-ERS voltohmmeter and Endohm chambers. Permeation experiments using ginger preparations were performed in independent triplicates using HBSS instead of culture medium.

Ginger Hot Flavor™ extract was added at 50 µg/ml either on the apical or basolateral side, respectively. After one and two hours of incubation at 37 °C the buffer on the opposite side was removed and replaced with fresh preconditioned one. After 3 hours, the buffer on both sides was removed and the compartments were washed with preconditioned buffer. Washing buffer was combined with the corresponding 3 hour sample. Monolayers were removed with a rubber policeman and extracted with 96 % EtOH. The 1, 2, and 3 hour ‘opposite side’ samples and the 3 hour ‘application side’ samples were lyophilised and the cell extract dried under a dry nitrogen flow. Lyophilisates were extracted with Et_2O, filtered, and dried. The samples were dissolved in DMSO and measured by HPLC with gradient C (chapter 3.8.4) at detection wavelengths of 210 and 280 nm. The percentages of absorption were calculated from linear calibration curves generated by serial dilution of the ginger Hot Flavor™ extract in HBSS and corresponding workup.

3.8.7 Co-culture Model

Cells were gathered at around 80 % confluence (~$8 \cdot 10^4$ to $1 \cdot 10^5$ cells/cm^2), seeded on Transwell-COL membrane inserts (24 well plate) at a density of $1 \cdot 10^5$ cells/ml, and cultured for two days resulting in a monolayer. 1 mM sodium butyrate was added and the monolayers incubated for five days to reach a TEER either of around 200 Ω/cm^2 or exceeding 400 Ω/cm^2 determined at 37 °C using the Millicell ERS-1 system.

Experiments were performed in one volume part HBSS containing 10 % autologous serum on the apical side and four volume parts fresh drawn human whole blood diluted 1:1 with HBSS on the basolateral side. At least three donors and three different Caco-2 passages were used in duplicates.

An AGP containing ethanolic extracts of ginger, the ginger Hot Flavor™ extract, and an aqueous carrot extract were tested at 50 µg/ml added to the apical side and incubated for 18 h. In case of the ginger Hot Flavor™ extract the blood was additionally stimulated with 15 ng/ml PMA and 1µl/ml αCD3. The extracts (at 10 and 50 µg/ml) were also tested in whole blood diluted 1:1 with HBSS as positive controls according the procedure in chapter 3.10.1. Cytokines in both compartments were quantified with the BD human inflammation kit according to the aforementioned procedure.

3.8.8 IL-1β Quantification

For IL-1β quantification with the CBA technique, monocytes were seeded in 96 well plates at $2 \cdot 10^5$ cells per well and incubated for at least 1 hour (sufficiently long to attach to the bottom). Cells were stimulated for 4 h under different priming conditions (vehicle, 100 ng/ml LPS and/or 10 µg/ml ginger Hot Flavor™ extract), spun down at 200g for 5', and medium was replaced (to be processed as described below) with 100 µl fresh one containing vehicle, 1 mM ATP, and/or 10 µg/ml ginger Hot Flavor™ CO_2 supernatant were removed. Medium samples and cells were shock frozen and lyophilised to be stored at -80 °C. Lyophilisates were reconstituted in 10 µl reconstitution buffer (10 mM HEPES, 0.3 mM EGTA, 0.1 % Triton® X-100, adjusted with KOH to pH 7.4 at RT). IL-1β was quantified using the IL-1β FlexSet according to the manufacturer's recommendation as described in chapter 3.8.3 with minor differences in point 1: Capture Beads were vortex, diluted and 10 µl diluted Capture Beads were added to all assay tubes. Data analysis was done with the FCAP™ Array Software.

3.8.9 U-937 Differentiation

U-937 cells were evaluated for their suitability as alternative model to whole blood and primary monocytes. Untreated isolated monocytes (grown for seven days in culture medium) were compared to MΦ (U-937 monocytes differentiated with 10 nM PMA for seven days). Each $1 \cdot 10^5$ cells per well were stimulated with 2 µg/ml LPS or 50 µg/ml soluble heat-aggregated IgG complexes (haIgG). Cytokine expression was determined after 3, 6, 9, and 24 hours using the BD Human Inflammation Kit.

3.8.10 $P2X_7$ Receptor Function

$Ethidium^+$ uptake was measured in isolated monocytes according to Jursik et al. [325] in 'K medium' without cell-markers.

Isolated monocytes were thawed, suspended with RPMI-1640 medium and spun down at 200g for 3'. For each sample, $1 \cdot 10^6$ cells were diluted in 0.3 ml 'K medium' (150 mM KCl, 10 mM HEPES, 5 mM glucose, 0.1 % BSA adjusted to pH 7.4 at 37 °C with KOH). Vehicle control and ginger Hot Flavor™ extract (10 µM), respectively, were added and the samples incubated for 15 min. Ethidium bromide (25 µM) was added and an aliquoted measured by flow cytometry (negative control). To a second aliquot, 1 mM ATP was added and kinetic flow cytometry measurements immediately started.

Data of the $ethidium^+$ fluorescence were exported as listmode files and plotted as time versus mean fluorescence graphs. As no differences were visible between vehicle and ginger treated cells further mathematic evaluations could be omitted.

3.8.11 Arachidonic Acid, PGE_2, and Palmitoylethanolamide in Macrophages

Free arachidonic acid, PGE_2, and palmitoylethanolamide (PEA) levels in U-937 MΦ and their culture medium were measured at the Institute of Biochemistry and Molecular Medicine, University Bern, Switzerland.

In short: $5 \cdot 10^6$ U-937 were cultured in complete RPMI medium and differentiated with 2 nM PMA for 48 h and then washed with PBS. The medium was replaced with FBS free one and the cells were incubated with test compounds (DMSO, 2 µM 10-S, 2 µg/ml ginger Hot Flavor™ extract, 50 and 100 µM acetyl salicylic acid, 0.5 µM MAFP) and incubated. After 30 min. LPS (1 µg/ml) was added and the cells incubated for another 4 h. Cells were scrape off and separate from the supernatant by centrifugation at 1000 rpm for 5 min.

Lipids were separately extracted from the cellular fraction and the supernatants, respectively, as follows: Cells were extracted according the Folch lipid extraction and the two fractions purified by solid phase extraction as follows. Cell pellets were sonicated for 5 min at 4 °C ice-cold chloroform (1 ml containing the internal standards), methanol (0.5 ml), and PBS (0.25 ml) and then centrifuged for 5 min at 800 g. The organic phase was dried in a glass vial and dry under N_2 and reconstituted in 1 ml ethanol by vortexing at RT (<5 min), diluted with 9 ml water. 1 ml ethanol was added to internal standards (*vide infra*) and mixed with 9 ml supernatant. Both fractions were then extracted by solid-phase extraction; the pH was brought to 3 with hydrochloric acid and the samples applied to C 18 Sep-Pak cartridge (Waters) (pre-activate with 3 ml of methanol and equilibrate with 3 ml 10 % ethanol). Cartridges were washed with 10% ethanol and eluted with 3 ml acetonitrile / ethyl acetate (1:1). Samples were finally evaporated to dryness.

Internal standards: PGE_2-d4 4ng/ul (100 ng/sample), AA-d8 4 ng/ul (100 ng/sample), and PEA-d5 1ng/ul (25 ng/sample).

The hydroxyl group of PEA and PGE_2 were then derivatised as described by Obata et al. [326], carbonic acid groups (e.g. arachidonic acid and PGE_2) according Balazy [327] and the ketone function of PGE_2 according Obata et al. [326] as follows. Derivatisation of the hydroxyl group of PEA was performed with the silylating agent dimethylisopropylsilyl (DMIPS) imidazole. As described by Obata et al. the molecular stability is increased and large fragments could be detected [326]. Derivatisation of arachidonic acid was achieved by esterification with pentafluorobenzylbromide (PFB bromide) and N,N-diisopropylethylamine [327]. The hydroxyl groups of PGE_2 were readily silylated with DMIPS imidazole and the carboxylic acid as for arachidonic acid with PFB bromide. The remaining ketone was transformed into an O-methyl oxime by methoxiyamine (MOX) hydrochloride in pyridine [326].

The samples were analyzed by GC/Electron Ionization (EI) mass spectrometry using an Agilent 6890N GC equipped with a 30 m HP-5MS column and a 5975C MS with triple-axis detector. As carrier gas helium was used at a constant flow rate of 1.5 ml/min with splitless injection at an inlet temperature of 250°C. Optimal separation of the 3 analytes was achieved with the following oven program: Initial temp. 150°C for 1 min followed by an increase to 280°C at 8°C/min with a final time of 20 min [328].

The ions summarized in Table 1 were used for selected ion monitoring.

	m/z quantifier Ion	m/z qualifier ion(s)	retention time
PEA	356	384	15.8
PEA-d5	361		
AA	386	303/346/484	15.9
AA-d8	392	350	
PGE_2	489	572/612/718	25.7 / 26.8
PGE_2-d4	493	576/616/722	

TABLE 1: Ions used for SIM and corresponding retention times

The retention times for the deuterated internal standards were few seconds shorter than for the corresponding analyte. The chromatogram of PGE_2 showed two peaks at different retention times. This was due to the formation of syn-anti isomers during derivatisation with MOX. Peak areas were combined for all quantifications [329].

3.8.12 ELISA Measurements of Thromboxane B_2 and Leukotriene B_4

Thromboxane B_2 (TXB_2) and leukotriene B_4 (LTB_4) levels in the cell culture supernatants of U-937 MΦ were measured at the Institute of Biochemistry and Molecular Medicine, University Bern, Switzerland.

In short: $2 \cdot 10^6$ U-937 were stimulated with 1 µg/ml LPS for 3 hours to measure TXB_2 and $10 \cdot 10^6$ primary $CD14^+$ monocytes were co-stimulated with 1 µg/ml LPS and 1 µM fMLP for 3 hours to measure LTB_4. TXB_2 and LTB_4 were quantified using colorimetric ELISA kits from Cayman Chemicals (100004023) and from Thermo Scientific (EHLTB4), respectively [328].

3.8.13 Calcium in Jurkat Cells

Calcium release from intracellular stores was measured by flow cytometry in Jurkat cells stained with Fluo-3. The assay procedure was similar to that described by Vandenberghe and Ceuppens [330].

0: Stock solutions (*vide infra*) were prepared and media pre-equilibrated.

1. For each sample 10^6 cells/ml were incubated in 1 ml fresh RPMI 1640 medium for 30 min at 37 °C.
2. Vehicle controls or probes (1000 X in DMSO) were added and incubated for 1 hour.
3. The samples were centrifuged at 200 g for 2'.

4. The pellets were re-suspended in 0.5 ml working solution (Fluo-3 AM [4 µM], Pluronic F-127 [75 µg/ml], and 0.2 % DMSO in HBSS plus 1 % FCS) and incubated for 20 min at 37 °C.
5. The samples were diluted 1:5 with HBSS containing 1 % FCS and incubated for 40 min.
6. The samples were diluted 1:2 with HBSS and centrifuged at 200 g for 2'.
7. The pellets were resuspended in 8 ml HEPES buffered saline (*vide infra*) and centrifuged at 200 g for 2'.
8. The cells were resuspended at $4 \cdot 10^6$/ml in HEPES buffered saline (*vide infra*) and incubated for 2.5 min at 37 °C.
9. The cells were stimulated with αCD3 Σ-chain (0.1 or 1µl/10^6 cells) and immediately measured with FACScan over time for 1.4 min with a resolution of 100 ms.

CAVE: The single samples were treated in a seven minute interval to guarantee an absolutely identical timeframe.

HEPES Buffered Saline: 137 mM NaCl, 10 mM HEPES, 5 mM KCL, 5 mM glucose, 1 mM Na_2HPO_4, 0.5 mM $MgCl_2$, 0.1 mM EGTA, and 0.1 % BSA, adjusted with NaOH to pH 7.4 at 37 °C.

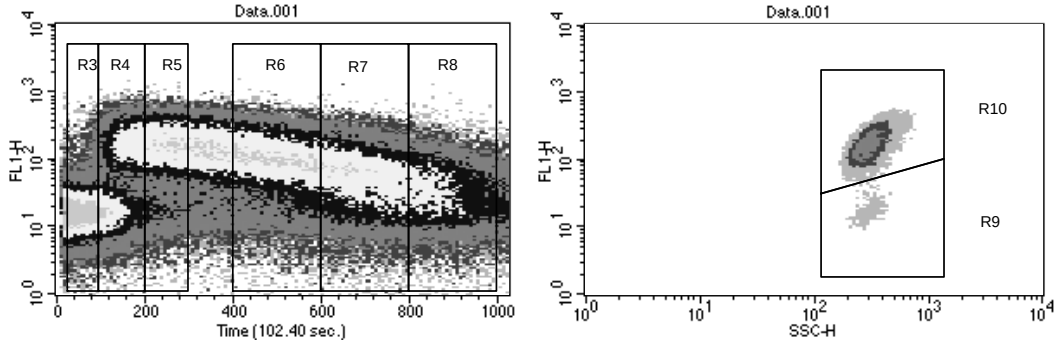


Figure 10: Gating of Jurkat cells labelled with the fluorescent calcium dye Fluo-3.

Left: Time (x-axis in 1/10 seconds) versus Fluo-3 fluorescence intensity (y-axis in arbitrary units). Cells were stimulated with αCD3 Σ-chain (1µl/10^6 cells) directly before the measurement. For quantification the 10 seconds of maximum fluorescence (gate R5) were used. Right: Gate R5 was plotted as sideward scatter (SSC, x-axis in arbitrary units) versus fluorescence intensity (y-axis in arbitrary units). The quotient of the mean fluorescence intensity of the high-fluorescent (R10) versus the low-fluorescent (R9, correlates with unstimulated cells) population was used for quantification.

Induction was calculated by considering the 10 seconds of maximal fluorescence (gate R5, left of Fig. 10). The geometric mean of the induced population (gate R10, right of Fig. 10) was divided trough the non-induced one (gate R9, right of Fig. 10). The quotient of the vehicle control was set as 100 %.

3.8.14 MAP Kinase Phosphorylation

MAP kinase phosphorylation in leukocytes was quantified using BD FlexSets according to the manufacturer's recommendations but with five times smaller volumes.

First, protein concentration was determined as follows. Lymphocytes, isolated from buffy coats, were adjusted to $2 \cdot 10^6$ cells/ml, Jurkat cells to $5 \cdot 10^5$ cells/ml, and isolated human monocytes to $1.5 \cdot 10^6$ cells/ml (corresponding each to a protein concentration of around 50 µg/ml). 1 ml thereof was centrifuged at 300 g for 3' at 4 °C. The pellet was lysed with 50 µl 1:4 diluted BD Denaturation Buffer and boiled for 5' followed by sonication and vigorous pipetting resulting in a clear solution. 25 µl were used to determine the protein concentration with the BioRad assay.

Quantification of phosphorylated MAP kinases in lymphocytes was done as follows. Isolated human lymphocytes were adjusted to $4 \cdot 10^6$ cells/ml and Jurkat cells to $1 \cdot 10^6$ cells/ml and aliquoted at 50 µl into 96-well PCR plates. 50 µl diluted stimuli were added as 2 X stock and incubated at 37 °C for the indicated time. Optimal conditions were for p38 phosphorylation: 10 µl/ml αCD3 Σ-chain and 2 nM PMA for 20'; for ERK1/2 phosphorylation: 10 nM PMA for 30'; and for JNK1/2 phosphorylation: 10 µl/ml αCD3 Σ-chain and 10 nM PMA for 60'. Subsequently, samples were cooled on ice and centrifuged at 300 g for 3' at 4 °C. The pellets were lysed with 5 µl diluted BD Denaturation Buffer containing phosphatase inhibitor cocktail and heated to 80 °C for 5'. Then the samples were cooled on ice, diluted with 5 µl assay diluent, and shock frozen in liquid nitrogen. After re-thawing, the lysates were vigorously pipetted to give a clear solution. Finally, phosphorylation was determined with BD FlexSets as described in chapter 3.8.8 (new formulations).

Jurkat cells and monocytes were used in small screenings but were not further examined.

3.8.15 Lymphocyte Proliferation

Lymphocytes, isolated from buffy coats, were seeded in 96-well plates at $1 \cdot 10^6$ cells/ml and 100 µl/well. 50 µl RPMI-1640 medium containing 0.625 µl BrdU stock solution and 2 µl αCD3 Σ-chain per ml and 50 µl RPMI-1640 medium containing vehicle control or test compounds were added and incubated for five days.

Proliferation was determined with the BD FITC BrdU Flow Kit according to the manufacturer's recommendations [331] with minor modifications as follows.

Where not indicated differently, incubation and centrifugation was performed at 4 °C.

0: Reagents were prepared: Perm/Wash buffer stock was diluted 10 times with water; staining buffer was diluted 33.3 times with water; DNAse was diluted 3.33 times with PBS; αBrdU was diluted 50 times with Perm/Wash buffer; human IgG and αCD3 were each diluted 50 times with staining buffer; secondary antibody ($F(ab')_2$ fragment against mouse IgG, PE conjugate) was diluted 10 times with staining buffer.

1: Cells were vigorously pipetted to disintegrate cell aggregates and transferred into a 96-well PCR plate and centrifuged at 250 g for 5 min at RT.

2: Supernatant was removed and cells were washed with 100 µl staining buffer and centrifuged at 250 g for 5 min at RT.

3: Optionally, cells were stained with αCD3 (if not, procedure was continued at point 5): Unspecific binding was reduced by adding 5 µl human IgG for 30' on ice followed by adding 5 µl αCD3 for 30 min on ice. Cells were washed with 100 µl staining buffer and centrifuged at 250 g for 5 min.

4: Supernatant was removed and cells were suspended in 10 µl secondary antibody for 30' on ice. 100 µl staining buffer was added and centrifuged at 250 g for 5 min.

5: Supernatant was removed and cells were suspended in 10 µl Cytifix/Cytoperm for 30 min on ice. 100 µl Perm/Wash buffer was added and centrifuged at 250 g for 5 min.

6: Supernatant was removed and cells were suspended in 10 µl Cytoperm Plus for 10 min on ice. 100 µl Perm/Wash buffer was added and centrifuged at 250 g for 5 min.

7: Supernatant was removed and cells were suspended in 10 µl Cytifix/Cytoperm for 5 min on ice. 100 µl Perm/Wash buffer was added and centrifuged at 250 g for 5 min.

8: Supernatant was removed and cells were suspended in 10 µl DNAse for 60 min at 37°C. 100 µl Perm/Wash buffer was added and centrifuged at 250 g for 5 min at RT.

9: Supernatant was removed and cells were suspended in 5 µl Perm/Wash buffer containing αBrdU for 20 min at RT. 100 µl Perm/Wash buffer was added and centrifuged at 250 g for 5 min at RT.

10: 2 µl 7-AAD were added and incubated for >5 min at RT.

11: 100 µl staining buffer was added and incubated for >5 min at RT.

12: Cells were analyzed by flow cytometry and CellQuest™ software version 3.3.

3.8.16 Cytotoxicity Assay

Cytotoxicity in all primary cells and cell lines was routinely monitored by microscopy and trypan blue exclusion and in blood by Wright-Giemsa staining.

Cytotoxicity of Yariv's reagent was determined in Caco-2, KB, CHO CB2, and MCF7 cells treatment for 72 hours. Morphology and adhesion/detachment were evaluated by microscopy. Cells were stained with 0.5 % methylene blue in PBS and the absorption at 650 nm was measured. Yariv's reagent had no effect at concentrations up to 50 µM. Besides, the concentration of Yariv's reagent could be verified by measuring the absorption at 405 nm.

All 8- and 10-homologues of the compounds isolated from ginger rhizome were toxic in serum free media above 10-20 µM for isolated human lymphocytes and Jurkat cells. They caused drastic morphological changes (confirmed by FACS analysis) and above 50 µM cell lysis within minutes. On the other hand, in complete culture medium containing 10 % FCS concentrations below 10 µM were well tolerated over at least three days.

Damaging effects on Caco-2 cells could be monitored by TEER. 50 µg/ml of the ginger Hot Flavor™ extract were tolerated for three hours in serum free buffer and for at least one day in complete culture medium. Higher concentrations caused a drop in TEER within minutes and detachment from the surface within one hour.

Viability determination with the vital dye WST-1 was not suitable in combination with ginger constituents.

3.8.17 P-glycoprotein activity

P-glycoprotein (Pgp or MDR1) activity was determined by Rhodamine123 (Rh123)-efflux, a fluorescent Pgp substrate, using human Pgp transfected MDCK cells (kindly obtained from Dr. Stefanie Krämer, ETH Zurich, Switzerland). Briefly, cells ($1 \cdot 10^6$ cells/tube) were incubated with Rh123 (Sigma, St. Louis, USA) at a final concentration equal to 500 µM for 20 min at 37 °C. Following centrifugation, cells were washed and

incubated with Rh123-free medium, in the absence or presence of test compounds at final concentrations of 10 and 30 μM for 1.5 h, at 37 °C. The fluorescence was measured using a FACScan (Becton Dickinson, USA) and the CellQuest software. Verapamil and vinblastine (both from Fluka, Switzerland) were used as positive controls. To validate the assay, untransfected MDCK cells were used which showed significantly less efflux of Rh123 over 1.5 h. [332]

3.9 Radioligand Assays

3.9.1 Radioligand Displacement Assays

Displacement assays were performed with commercial membrane preparations over-expressing the respective receptors. Radioligand displacement assays at the human 5-HT_{1A} and 5-HT_3 receptors were done according to the manufacturers recommended procedure using the following buffers. 5-HT_{1A}R: 50 mM TRIS, 5 mM $MgSO_4$ and 5-HT_3R: 50 mM TRIS, 5 mM $MgCl_2$, 1 mM EDTA, both adjusted with HCl to pH 7.4 at 37 °C.

In short: 100 μl incubation buffer was mixed with 40 μl [^{3}H]8-OH-DPAT [20 nM], 20 μl diluted membranes (1:20 dilution in incubation buffer, homogenized using a Dounce grinder), and 40 μl of samples. The latter contained incubation buffer, ginger extracts, ginger extract fractions (solutions in incubation buffer, for screening reasons at a final concentration of 25 μg/ml and 0.5 % DMSO), or serotonin [50 μM] for non-specific binding. The solution was incubated for two hours at 37 °C in the dark. Then filtered over glass fibre filter type pcs G-7 pre-soaked in 0.5 % polyethylenimine, and washed 3 times with ice cold 50 mM TRIS-HCl pH 7.4. The filter spots were transferred to 3 ml scintillation cocktail, shaken on a shaker KS10 for one hour, and measured with a Scintillation Counter.

All experiments for screening reasons were done ones in a duplicate and measurements for the determination of K_i values were done tree times each in a triplicate. The K_i values were calculated using the Cheng-Prussof equation [333] based on Hill plots as reported by [321]. Test compounds were stored as 5 mM stock solutions in DMSO at -80 °C.

3.9.2 G_i activity using [^{35}S]GTPγS

[^{35}S]GTPγS binding was determined with membrane preparations of the HeLa cell line HA6 which has a $G\alpha_i$ coupled signal transduction [334].

Sub-confluent cells were resuspended and vortexed in ice-cold buffer A (50 mM TRIS, 200 mM NaCl, 10 mM EGTA, 3 mM EDTA, and 0.1 % Protease Inhibitor Cocktail, adjusted with HCl to pH 7.6 at 4 °C) followed by centrifugation at 40'000 g for 25 min at 4 °C. The pellet was re-suspended in buffer B (50 mM TRIS, 200 mM NaCl, 1 mM EGTA, 0.3 mM EDTA, and 0.01 % Protease Inhibitor Cocktail, adjusted with HCl to pH 7.6 at 4 °C), briefly sonicated, homogenized with a Dounce homogenizer (10 strokes), and centrifuged at 40'000 g for 25 min at 4 °C. The pellet was re-suspended in freezing buffer (50 mM TRIS, 100 mM NaCl, 0.3 mM EDTA, 10 mM magnesium acetate, 0.2 mM DTT, and 10 % sucrose, adjusted with HCl to pH 7.6 at 4 °C) at a protein concentration of 1.5 to 3 mg/ml determined with the BioRad protocol, and stored at -80 °C.

2.5 µl membrane preparation were diluted in 87.5 µl incubation buffer (20 mM MOPS, 10 mM magnesium acetate, 30 µM GDP, 0.2 mM DTT, adjusted with KOH to pH 7.4 at 30 °C) containing vehicle, positive control (10 µM 8-OH-DPAT), or test compounds, and incubated for 20 min at RT. The reaction mixture was cooled down to 4 °C for 15 min. The reaction was started by addition of 10 µl GTPγS [1 nM] (final concentration: 98 pM cold and 2 pM hot GTPγS) and incubation for 30 min at 30°C. The reaction was stopped by cooling to 4°C, filtered over a Whatman GF/B glass fibre filter pre-soaked in wash buffer (*vide infra*) containing 1 mM ATP, and washed 3 times with wash buffer (20 mM HEPES, 10 mM magnesium acetate, 10 µM ATP, 0.2 mM DTT, adjusted with KOH to pH 7.4 at 4 °C). The filters were transferred to 3 ml scintillation cocktail, shaken on a shaker KS10 until complete disintegration, and measured with a scintillation counter.

Non-specific binding was determined with additional 10 µM cold GTPγS added to the incubation buffer. Test compounds (5 mM in DMSO) and reagents (in GDP-free incubation buffer) were stored at -80 °C. All experiments were done at least in triplicates with different membrane preparations.

Reported [^{35}S]GTPγS assay conditions for $G\alpha_i$ coupled GPCR (for example [334]) use often similar conditions and were first tested but subsequently optimized as follows.

Cell lysis and membrane isolation: 15 mM TRIS HCl was changed to 50 mM TRIS HCl containing 200 mM NaCl and 0.01% Protease Inhibitor Cocktail

Incubation buffer: 20 mM HEPES were replaced with 20 mM MOPS and therefore the 0.1% ascorbic acid had to be replaced by 0.2 mM DTT because the former degrades MOPS. Alternatively, MOPS may be stabilized with 0.1 mM sorbitol.

To reduce constitutive activity no NaCl was used and $MgCl_2$ was replaced with magnesium acetate. Better results were also obtained by replacing NaOH with KOH. Finally, hot GTPγS was diluted with cold one to reduce mCi/ml.

The following substances were unsuccessfully tested for altering solubility of ginger extracts: BSA, 1% DMSO, 0.5% polidocanol (Lubrol™)

Wash buffer: 10 μM ATP were added to reduce nonspecific binding. Unsuccessful additives were 0.1% ascorbic acid and 0.1% polidocanol (Lubrol™)

Glass fibre filter: Type pcs G-7 pre-soaked in polyethylenimine were replaced with Whatman GF/B pre-soaked in 1 mM ATP markedly increasing membrane and specific binding and drastically reducing nonspecific binding. Unsuccessful were nitrocellulose membrane, glass fibre filter pre-soaked in 0.1% polidocanol (Lubrol™) or Tween-20, and silanized type pcs G-7 glass fibre filters.

3.10 SDS-PAGE and Western Blot

3.10.1 SDS-PAGE Lysis Protocol

5 μl modified RIPA buffer (see 3.6.3) were used for the lysis of $2 \cdot 10^5$ cells followed by heating to 70 °C for 5 min. in a water-bath. The lysate was dilute with 5 μl of appropriate assay diluent (e.g. by BD Biosciences) and frozen at -80 °C or immediately processed according to the manufacturer's protocol.

Electrophoresis: 150 V, usually for 80'

3.10.2 Western Blot and Staining

Blotting was done according to the NOVEX® Invitrogen protocol.

Staining was done according to the ECL manual from PerkinElmer using TBS-Tween (TRIS-buffered saline with 0.1 % Tween 20) adjusted to pH 7.6 at the used temperatures.

Nitrocellulose transfer membranes were blocked with TBS-Tween plus 5 % defatted milk powder. Antibody labelling was done in TBS-Tween containing 1 % defatted milk powder. Primary antibodies against IL-1β (pro and mature form) were diluted 1:250, primary antibodies against phosphor-p38, phosphor-JNK, and CD3 ε-chain each 1:1'000, secondary antibodies against mouse IgG 1:8'000, and secondary antibodies against rabbit IgG 1:2'000.

3.10.3 Quantification

Nitrocellulose membranes were developed according to the Amersham Biosciences recommendations using ECL Plus Western Blotting Detection Reagent. Photos were taken in a dark chamber with transparent Kodak photographic paper. Photos were scanned, digitalized, and evaluated with the ImageJ program.

3.10.4 MAP Kinase Phosphorylation

The procedure was similar as described in chapter 3.8.11 but with $7.5 \cdot 10^5$ cells/ml instead. Conditions were for p38: 37.5 µl/ml αCD3 Σ-chain and 2 nM PMA for 20' and for JNK1/2: 37.5 µl/ml αCD3 Σ-chain and 10 nM PMA for 60'. Cells were lysed with 30 µl modified RIPA buffer.

3.10.5 Interleukin 1β

IL-1β detection by SDS-PAGE and Western blot was similar to the CBA procedure described in chapter 3.8.7. Monocytes were stimulated with 100 ng/ml LPS in fresh RPMI-1640 medium for 4 h. Then, the medium was replaced by gluconate Basal Salt Solution (according the method used by Qu et al. [146] but with minor modifications: 130 mM sodium gluconate, 20 mM HEPES, 5 mM KCl, 5 mM glucose, 1.5 mM $CaCl_2$, and 1.0 mM $MgCl_2$, adjusted with NaOH to pH 7.5 at 37 °C). Further processing was in analogy to the CBA procedure except that lyophilisates were dissolved in 10 µl sample buffer (6.5 parts water, 2.5 parts NuPage® LDS buffer, and 1 part NuPage® antioxidant). The IL-1β (1 ng/ml) and the pro-IL-1β standard were diluted ten times with water and 1 µl of both were diluted in 8 µl sample buffer.

3.11 Assaying Phospholipases A_2

3.11.1 Isolation of Phospholipids

Crude phosphatidylcholine (PC) was isolated from commercial grade lecithin by quantitative de-oiling with acetone, enrichment of PC by quantitative extraction with boiling absolute EtOH and finally precipitation of impurities (e.g. glucosylceramide) at 0 °C [335]. Crude PC was purified by solid-phase extraction using silica: Residual apolar lipids were removed with EtOH/AcOH (95:5) and PC selectively washed out with DCM/MeOH/AcOH 5:5:1 whilst lyso-derivatives remained on the column. PC was dried, neutralized by filtration over sodium carbonate and anhydrous magnesium sulphate, stabilized with 10 ppm α-tocopherol [336], dried, and stored at -80 °C. The PC fraction had a TLC purity of more than 95 %.

Lyso-PC derivatives were synthesised by hydrolysis of SUV (see chapter 3.16.5) using $sPLA_2$ from hog pancreas. They were isolated with an improved Folch lipid extraction as described by Matyash et al. [337]. In short: for each 200 µl reaction mixture 1.5 ml MeOH were added and the sample vortexed. 5 ml MtBE were added and shaken for 1 hour. Finally 1.25 ml water was added and kept for 10 min at RT. Phases were separated by centrifugation at 1'000 g for 10 min, the upper phase (containing the lipids) was removed, and lower phase was re-extracted with 2 ml of freshly prepared upper phase. The joined upper phases were dried under a dry nitrogen flow and stored at -80 °C. Stocks were prepared in $CHCl_3$/MeOH (95:5).

3.11.2 Phospholipase A_2 Isolation

Phospholipase A_2 was enriched from U-937 cells cultured at approximately 10^6 cells/ml. Cells were stimulated with 35 ng/ml PMA for 24 h according to published protocols [338, 339].

$2.8 \cdot 10^7$ cells were lysed in hypotone lysis buffer (340mM sucrose, 10mM HEPES, 1mM EDTA, 1mM DTT, adjusted with KOH to pH 7.4 at 4 °C) according to Ackermann et al. [340] and centrifuged at 1'000 g at 4 °C for 30 min. The supernatants were concentrated by ultrafiltrated with Vivaspin 15 (50 kDa MWCO) resulting in a protein concentration (determined with the BioRad protocol) of 0.625 to 1.5 mg protein/ml.

3.11.3 Assay Buffers and Lipid Preparations

Stock solutions of palmitatoyl-6-*O*-ascorbate potassium salt (PAK) were prepared in $CHCl_3$, all others in EtOH, and were mixed and dried under a nitrogen flow directly before micelle preparation (Table 2). The mixed lipid film was hydrated in corresponding mixed micelle buffer at 40 °C (Table 2), if necessary briefly sonicated to detach film residues from the glass surface, and gently (to avoid foam formation) shaken until the solution became completely clear.

Size and zeta potential of the micelles were analyzed on a Zetasizer™. One single sharp peak with an average size distribution of 32.3 ± 19.2 nm by volume (area of 99.3 %), 27.9 ± 12.5 nm by number (area of 100 %), and an average zeta potential of -39.4 ± 5.3 was obtained (Fig. 17 chapter 4.2.2).

PLA_2 class	$iPLA_2$	$cPLA_2$	$sPLA_2$
Enzyme preparations[1)]	340mM sucrose, 10mM HEPES, 1mM EDTA, 1mM DTT, adjusted with KOH to pH 7.4 at 4 °C	340mM sucrose, 10mM HEPES, 1mM EDTA, 1mM DTT, adjusted with KOH to pH 7.4 at 4 °C	3.2 M (NH4)2SO4, pH 5.5
Substrate buffer conditions[1)]	190 mM MOPS, 152 mM KOH, 3 mM DTT, 2 mM ATP, 1 mg/ml BSA	190 mM MOPS, 152 mM KOH, 3 mM DTT, 1.2 mM $CaCl_2$, 1 mg/ml BSA, 20 µM BEL	100 mM MOPS, 100 mM NaCl, 1 mM $CaCl_2$, and 1 mg/ml BSA adjusted to pH 7.5 with KOH
Substrate, final concentration	8 mM PAK, 1 mM Tween 80 and 1 mM thio-PC[2)] mixed micelles self forming at T > 35 °C [341]	8 mM PAK, 1 mM Tween 80 and 1 mM thio-PC[2)] mixed micelles self forming at T > 35 °C [341]	0.8 mg/ml PC[3)], 50 µM DSPA, 50 µM PAK, 1 µM α-tocopherol
Test compounds, final concentration	10 µg/ml ginger Hot Flavor™ extract, 10 µM pure ginger constituents, 10 µM BEL, 10 µM MAFP	10 µg/ml ginger Hot Flavor™ extract, 10 µM pure ginger constituents, 10 µM MAFP, 1 mM EDTA	10 µg/ml ginger Hot Flavor™ extract

Table 2: Conditions for the phospholipase A_2 assay.

[1)] For i- and $cPLA_2$, the concentrations in the final reaction mixture consisted of ½ enzyme preparation and ½ substrate buffer.

[2)] During assay optimisation umbelliferyl arachidonate was used instead of thio-PC.

[3)] Depending on the use, 1 mg/ml PC was replaced by 1 mM thio-PC or umbelliferyl arachidonate.

A 10 X stock of small unilamellar vesicles (SUV) was prepared with ~ 9 mM lipid substrate (either 7.2 mg/ml pure isolated PC or a mixture of 6.48 mg/ml thereof with 0.9 mM thio-PC or 0.9 mM umbelliferyl arachidonate, respectively), 500 µM DSPA, 500 µM PAK, and 10 µM α-tocopherol. The mixture was drying under rotation in a dry nitrogen flow, the resulting film rehydrated in SUV buffer, and sonicated until a clear and slightly opalescent solution was obtained. The lipid substrates were chosen depending on the purpose.

3.11.4 Assay for Intracellular Phospholipases A_2

Phospholipase A_2 preparation and mixed micelles (each 10 µl/sample) were mixed at RT giving a final concentrations of 100 mM Good's Buffer, ~ 80 mM KOH (pH of 7.4 at 40 °C), 170 mM sucrose, 2 mM DTT, 0.5 mg/ml BSA, 8 mM PAK, 1 mM Tween 80, 1 mM thio-PC, and either 1 mM ATP and 0.5 mM EDTA ($iPLA_2$) or 0.1 mM free calcium and 10 µM BEL ($cPLA_2$) (Table 2). Notably, a ten times lower mixed micelle concentration, still over the CMC [341], is sufficient for catalysis and fluorescence detection but exert a lower signal to noise ratio.

The reaction mixture was heated to 40 °C to give a clear solution and was incubated with frequent gentle shaking. The enzymatic reaction was stopped after 3 hours (over 50 but below 100 % hydrolysis) with 20 µl ice cold quenching solution (1 mM DTT, 1 mM Cs_4EDTA, and 20 mM Cs_2CO_3 in 88 % MeOH) and incubated for 1 hour at RT [342].

20 µl monobromobimane (10 mM Stock in MeOH) were added and incubated in the dark for hour at RT [343]. The reaction was stopped and stabilized by acidification with 1 µl trichloro acetic acid (2.2 M in water, resulting in a pH < 3). Samples were centrifuged at 500 g for 5' and the supernatant was removed and stored without decomposition for up to 3 days at -18 °C [344].

Positive control (100 % conversion) was mimicked by mixing 1 mM thio-PC, 10 mM mBrB, 200 mM hydroxylamine HCl, and 200 mM sodium hydroxide for 15 min (adapted from [345]). The reaction was quenched by the addition of 4 % AcOH. This solution was used for HPLC calibration.

For analytical HPLC 20 µl reaction mixture were separated on a Nucleodur Sphinx column on an HPLC device with fluorescence detector. A 15' gradient of 0.25 % aqueous NH_3/acetonitrile was used: isocratic at 30:70 for 1', gradient to 10:90 during 8', isocratic for 2', gradient back to 30:70 during 2', isocratic for 2'. Detection parameters were: λ_{ex} 385nm and λ_{em} 485nm. The product had an R_f of 6'.

Enzymatic activity was calculated from the AUC as absolute [total product in nmol and nmol product/min/mg protein] and relative [% total conversion and % conversion compared to control] values.

3.11.5 Secretory Phospholipase A_2 Assay

Directly before use, 100 µl SUV (10 X) were diluted in 900 µl incubation buffer. 2 µl $sPLA_2$ from hog pancreas were added and incubation at 40 °C. Lipids were isolated with the improved Folch lipid extraction (see chapter 3.16.1). Preparations with thio-PC were dissolved in 200 µl THF/ET_3N (4:1), mixed with 2 µl 10 mM mBrB, and detected by TLC under UV at 366 nm. Preparations containing isolated PC or umbelliferyl arachidonate were separated by TLC and detected by molybdenum blue and basic polyethylenimine spray reagents, respectively.

3.12 Syntheses

3.12.1 10-Shogaol

9.57 mg isolated 10-G (fraction 7.16) were dissolved in 2 ml toluene and catalytic amounts of p-TosOH, refluxed under argon for four days, and regularly controlled by TLC with n-hexane/Et_2O/AcOH (20:20:1) as eluent. The product was separated by flash LC over 50 ml silica gel Si60 (40–65 µm) on a column with 1 cm diameter and pentane/Et_2O (1:1) as eluent. Fractions were monitored by TLC resulting in 1.78 mg 10-S, 0.97 mg 10-G, and 6.69 mg unidentified by-products. 10-S was purified by HPLC (gradient B, chapter 3.8.4) resulting in 0.74 mg product with a purity of > 95 % according to ^{1}H-NMR.

3.12.2 Yariv's Reagent

Synthesis of Yariv's reagent was similar to established procedures [346, 347] with minor modifications. 204 mg p-nitrophenyl-β-D-glucopyranose were dissolved in 20 ml MeOH and hydrogenated for three hours in a H_2 atmosphere in the presence of 21 mg Pd/C. TLC with n-butanol/MeOH/water (4:3:3) gave an R_f-value of 0.85 and 0.75 for the reagent and product, respectively. The product was solved in hot EtOH and re-crystallized at -20 °C over night to give 125 mg pure product (=62.5 % turn over).

125 mg p-aminophenyl-β-D-glucopyranose were dissolved in 2.76 ml 0.5 M HCl, 47.6 mg sodium nitrite in 1 ml water added dropwise, and the reaction stirred for one hour. Then, 14.5 mg phloroglucinol in 7.5 ml water were added dropwise over one hour under constant

stirring followed by the addition of a 1 M sodium hydroxide solution until pH 9.0 was reached and stayed constant for 1.5 hours. To the end-volume of 40 ml another 40 ml of EtOH were added. All steps were carried out at 4 °C. The precipitated product was separated from mono- and disubstituted phloroglucinol by filtration, washed with ice cold EtOH, and dried *in vacuo*. The filtrate was dried *in vacuo*, solved in 4 ml water, precipitated with 4 ml EtOH over night, filtered, dried *in vacuo*, and kept separately.
Structure and purity were confirmed by 2D-NMR.

3.12.3 Palmitoyl Ascorbate

Palmitoyl-6-*O*-ascorbic acid was synthesized according to Wen et al. [348] with minor modifications. Reaction conditions were as follows: 35 °C reaction temperature, 3.0 g ascorbic acid dissolved in 15 ml 95 % sulphuric acid, and 5.68 g palmitic acid, 100 % ultrasonic power output with stirring for 15'. The reaction mixture was neutralized with 25 g calcium carbonate in 100 ml ice water to give a slightly acidic pH. The subsequent workup was in accordance with the aforementioned authors. Identity and purity of the white waxy solid were confirmed by DC, ESI-MS, and 2D-NMR. The potassium salt was generated before use by mixing appropriate solutions of palmitoyl-6-*O*-ascorbic acid in $CHCl_3$ with KOH in MeOH to result in a 100 mM solution in 95:5 $CHCl_3$/MeOH.

3.12.4 Mosher's Ester of 6-Gingerol

42.1 mg 6-gingerol (fraction 11 from flash LC of the self-made ginger extract) in 429 µl tetrachloromethane were added to 50 mg (R)-(-)-Mosher's acid chloride in 437.8 µl pyridine (= 429 mg). The reaction was carried out under a nitrogen atmosphere and on ice. Then, the reaction was brought to RT and after one hour diluted with 2.5 ml Et_2O. The organic phase was washed with diluted HCl, saturated Na_2CO_3, and saturated NaCl solutions. The organic phase was dried over anhydrous $MgSO_4$ together with some silica gel. The powder was separated over a silica gel column with a solvent mixture of hexane:EtOAc (1:1) resulting in 2 fractions containing products and 4 fractions containing reagents. Mass spectroscopy showed disubstitution and ^{1}H-NMR a product mixture whereupon the two former fractions were subjected to another separation by column chromatography with a solvent mixture of hexane:EtOAc (2:1).

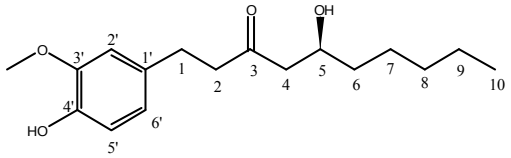
3.13 Characterization

3.13.1 ESI-MS

Characterization by molecular mass was done one an Alliance HT mass spectrometer using a solvent system of 30 % water with 0.1 % formic acid and 70 % acetonitrile. 20 µl of sample solution were injected. The mass range measured was m/z 100-500 and/or m/z 200-600, ESI (cone voltage): ± 20 eV, + 40 eV and + 80 eV, capillary voltage 3.0 kV, extractor 1 V and RF lens 0.2 V. Temperature was set 120 °C and desolvatation temperature at 250 °C. N_2 was used as carrier gas at a flow rate for desolvatation of 600 l/h and for cone flow of 40 l/h.

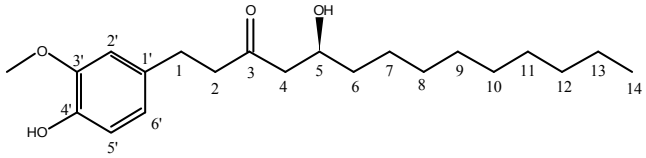
3.13.2 Characterisation of Isolated and Synthesized Compounds

6-Gingerol:



White crystals. > 95 % pure in HPLC. ^{1}H-NMR, 400 MHz, $CDCl_3$: δ = 0.86 (3H, t, 10-CH_3), 1.24-1.3 (4, m, CH_2CH_2), 1.3-1.5 (4H, t, 6,7-CH_2-CH_2), 2.48 (2H, d(dd), 4-CH_2) 2.69-2.7 (4H, dm, 1,2-CH_2-CH_2), 2.79 (1H, broad s, CHO<u>H</u>), 3.85 (3H, s, OCH_3), 4 (1H, m, C<u>H</u>OH), 5.46 (1H, s, arOH), 6.62-6.67 (2H, m, 2'-CH and 6'-CH), 6.8 (1H, d, 5'-CH). MS ES+, 8.5·e^6: m/z: 277 (6-shogaol +H_3O^+), 312, 317 (+Na^+).

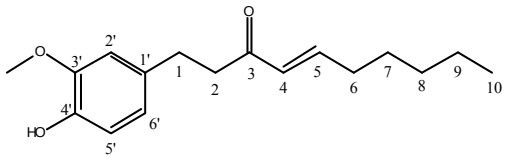
10-Gingerol:



White waxy crystals. > 95 % pure in HPLC. ^{1}H-NMR, 400 MHz, $CDCl_3$: δ = 0.83 (3H, t, 14-CH_3), 1.2-1.38 (12, m, $(CH_2)_6$), 1.38-1.7 (4H, t, 6,7-CH_2-CH_2), 2.48 (2H, dd, 4-CH_2) 2.68-2.82 (4H, dm, 1,2-CH_2-CH_2), 3.02 (1H, broad s, CHO<u>H</u>), 3.83 (3H, s, OCH_3), 4.00 (1H, m, C<u>H</u>OH), 5.79 (1H, broad s, arOH), 6.6-6.65 (2H, m, 2'-CH and 6'-CH), 6.79 (1H, d, 5'-CH). ^{13}C-NMR, 400 MHz, $CDCl_3$: δ = 14.1 (1C, s, 14-CH_3), 22.7 (1C, s, 13-CH_2), 25.5, 29.2, 29.3, 29.5, 29.55, 29.6, 31.9, (each 1C, s, 1,7,8,9,10,11,12-CH_2), 36.5 (1C, s, 6-CH_2), 45.4 (1C, s, 2-CH_2), 49.4 (1C, s, 4-CH_2), 55.9 (1C, s, -OCH_3), 67.7 (1C, s, 5-CHOH), 111 (1C, s, 2'-CH_2), 114.5 (1C, s, 5'-CH), 120.7 (1C, s, 6'-CH), 1302.6 (1C, 1, 1'-CH), 144

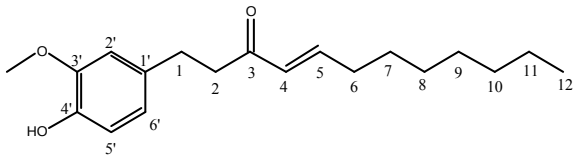
(1C, 1, 4'-CH), 146.6 (1C, s, 3'-C), 211.4 (1C, s, C=O). MS ES-, $7 \cdot e^5$: m/z: 313, 315, 349 (-H^+), 351.

6-Shogaol:



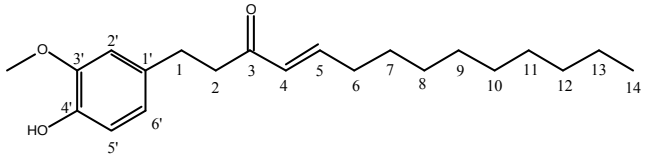
White waxy substance. > 95 % pure in HPLC. ^{1}H-NMR, 400 MHz, $CDCl_3$: δ = 0.88 (3H, t, 10-CH_3), 1.22-1.38 (4, m, CH_2CH_2), 1.44 (2H, t, 7-CH_2), 2.2 (2H, m, 6-CH_2), 2.85 (4H, m, CH_2-CH_2), 3.84 (3H, s, OCH_3), 5.48 (1H, broad s, arOH), 6.1 (1H, d, 4-CH), 6.7 (2H, m, 2'-CH and 6'-CH), 6.82 (2H, m, 5'-CH and 5-CH). ^{13}C-NMR, 400 MHz, $CDCl_3$: δ = 13.95 (1C, s, 10-CH_3), 22.42 (1C, s, 9-CH_2), 27.8, 31.3, 32.5 (each 1C, s, 7,6,8-CH_2), 42 (1C, s, 2-CH_2), 55.9 (1C, s, -OCH_3), 111.1 (1C, s, 2'-CH_2), 114.3 (1C, s, 5'-CH), 120.8 (1C, s, 6'-CH), 130.3 (1C, 1, 4-CH), 133.3 (1C, 1, 1'-CH), 142.9 (1C, s, 4'-C), 146.4 (1C, 1, 3'-CH), 147.9 (1C, s, 5-C), 199.8 (1C, s, C=O). MS ES+, $2.7 \cdot e^6$: m/z: 277 (+H^+), 299 (+Na^+).

8-Shogaol:



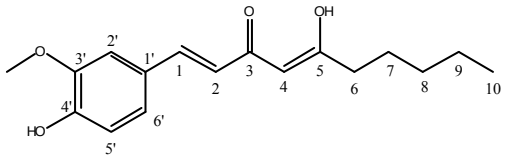
White waxy substance. > 95 % pure in HPLC. ^{1}H-NMR, 400 MHz, $CDCl_3$: δ = 0.8 (3H, t, 12-CH_3), 1.18-1.3 (8, m, $(CH_2)_4$), 1.38 (2H, t, 7-CH_2), 2.17 (2H, m, 6-CH_2), 2.79 (4H, m, CH_2-CH_2), 3.81 (3H, s, OCH_3), 5.4 (1H, broad s, arOH), 6.01 (1H, d, 4-CH), 6.62 (2H, m, 2'-CH and 6'-CH), 6.75 (2H, m, 5'-CH and 5-CH). ^{13}C-NMR, 400 MHz, $CDCl_3$: δ = 14 (1C, s, 12-CH_3), 23 (1C, s, 11-CH_2), 28, 29, 29.5, 32, 33 (each 1C, s, 6,7,8,9,10-CH_2), 42 (1C, s, 2-CH_2), 62 (1C, s, -OCH_3), 111 (1C, s, 2'-CH_2), 114 (1C, s, 5'-CH), 121 (1C, s, 6'-CH), 131 (1C, 1, 4-CH), 133 (1C, 1, 1'-CH), 143 (1C, s, 4'-C), 146 (1C, 1, 3'-CH), 149 (1C, s, 5-C), 200 (1C, s, C=O). MS ES+, $4.5 \cdot e^6$: m/z: 305 (+H^+), 327 (+Na^+).

10-Shogaol:



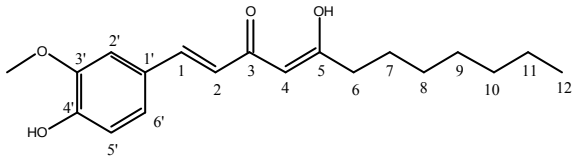
White crystalline wax. > 95 % pure in HPLC. ^{1}H-NMR, 400 MHz, $CDCl_3$: δ = 0.8 (3H, t, 14-CH_3), 1.16-1.3 (12, m, $(CH_2)_6$), 1.38 (2H, t, 7-CH_2), 2.17 (2H, m, 6-CH_2), 2.79 (4H, m, CH_2-CH_2), 3.81 (3H, s, OCH_3), 5.39 (1H, broad s, arOH), 6.01 (1H, d, 4-CH), 6.61 (2H, m, 2'-CH and 6'-CH), 6.68 (2H, m, 5'-CH and 5-CH). ^{13}C-NMR, 400 MHz, $CDCl_3$: δ = 13 (1C, s, 14-CH_3), 23 (1C, s, 13-CH_2), 28, 29.1, 29.2, 29.3, 29.5, 32, 33 (each 1C, s, 6,7,8,9,10,11,12-CH_2), 43 (1C, s, 2-CH_2), 62 (1C, s, -OCH_3), 111 (1C, s, 2'-CH_2), 114 (1C, s, 5'-CH), 121 (1C, s, 6'-CH), 131 (1C, 1, 4-CH), 133 (1C, 1, 1'-CH), 144 (1C, s, 4'-C), 147 (1C, 1, 3'-CH), 149 (1C, s, 5-C), 199.5 (1C, s, C=O). MS ES+, $1.0 \cdot e^{6}$: m/z: 333 ($+H^+$), 334 ($+2H^+$); ES-, $1.3 \cdot e^{4}$: m/z: 331 ($-H^+$), 367, 377, 394.

1-Dehydro-6-gingerdione:



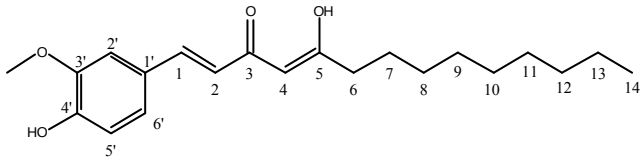
Light yellow wax, turns red in DMSO. > 95 % pure in HPLC. ^{1}H-NMR, 400 MHz, $CDCl_3$: δ = 0.93 (3H, t, 10-CH_3), 1.29-1.42 (4, m, CH_2CH_2), 1.63-1.73 (2H, m, 7-CH_2), 2.4 (2H, t, 6-CH_2), 3.96 (3H, s, OCH_3), 5.64 (1H, s, 4-CH), 5.84 (1H, s, arOH), 6.36 (1H, d, 2-CH), 6.94 (1H, d, 5'-CH), 7.04 (1H, d, 2'-CH), 7.11 (1H, dd, 6'-CH), 7.56 (1H, d, 1-CH). ^{13}C-NMR, 400 MHz, $CDCl_3$: δ = 13.92 (1C, s, 10-CH_3), 22.44 (1C, s, 9-CH_2), 25.3, 31.5, 40.1 (each 1C, s, 6,7,8-CH_2), 60.6 (1C, s, -OCH_3), 100.1 (1C, s, 4-CH_2), 109.4 (1C, s, 2'-CH), 114.8 (1C, s, 5'-CH), 120.6 (1C, 1, 2-CH), 122.6 (1C, 1, 6 '-CH), 127.7 (1C, s, 1'-C), 139.8 (1C, 1, 1-CH), 146.8 (1C, s, 4'-C), 147.6 (1C, s, 3'C), 178 (1C, s, 3-COH), 200.2 (1C, s, C=O). MS ES+, $8.0 \cdot e^{5}$: m/z: 271, 291 ($+H^+$), 371; ES-, $9.1 \cdot e^{3}$: m/z: 289 ($-H^+$), 353.

1-Dehydro-8-gingerdione:



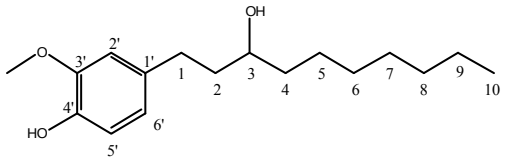
Yellow wax, turns orange in DMSO. > 95 % pure in HPLC. ^{1}H-NMR, 400 MHz, $CDCl_3$: δ = 0.9 (3H, t, 12-CH_3), 1.24-1.4 (8, m, $(CH_2)_4$), 1.63-1.71 (2H, m, 7-CH_2), 2.4 (2H, t, 6-CH_2), 3.96 (3H, s, OCH_3), 5.64 (1H, s, 4-CH), 5.84 (1H, s, arOH), 6.36 (1H, d, 2-CH), 6.94 (1H, d, 5'-CH), 7.04 (1H, d, 2'-CH), 7.11 (1H, dd, 6'-CH), 7.55 (1H, d, 1-CH). ^{13}C-NMR, 400 MHz, $CDCl_3$: δ = 12 (1C, s, 12-CH_3), 22 (1C, s, 11-CH_2), 25.6, 29.3, 31.7, 40.2, 41.6 (each 1C, s, 6,7,8, 9, 10-CH_2), 56.2 (1C, s, -OCH_3), 100.2 (1C, s, 4-CH_2), 109.3 (1C, s, 2'-CH), 114.7 (1C, s, 5'-CH), 120.7 (1C, 1, 2-CH), 127.4 (1C, 1, 6'-CH), 139.8 (1C, 1, 1-CH), 176.5 (1C, s, 3-COH). MS ES+, 5.5·e^5: m/z: 301 (-H_2O), 319 (+H^+), 371; ES-, 1.7·e^4: m/z: 317 (-H^+), 318.

1-Dehydro-10-gingerdione:



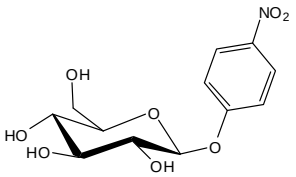
Yellow crystalline wax, turns orange in DMSO. > 95 % pure in HPLC. ^{1}H-NMR, 400 MHz, $CDCl_3$: δ = 0.9 (3H, t, 14-CH_3), 1.24-1.39 (12, m, $(CH_2)_6$), 1.63-1.70 (2H, m, 7-CH_2), 2.39 (2H, t, 6-CH_2), 3.96 (3H, s, OCH_3), 5.64 (1H, s, 4-CH), 5.84 (1H, s, arOH), 6.36 (1H, d, 2-CH), 6.94 (1H, d, 5'-CH), 7.04 (1H, d, 2'-CH), 7.11 (1H, dd, 6'-CH), 7.55 (1H, d, 1-CH). ^{13}C-NMR, 400 MHz, $CDCl_3$: δ = 14.2 (1C, s, 14-CH_3), 22.4 (1C, s, 13-CH_2), 25.8, 29.5, 29.52, 29.6, 29.62, 31.9, 40.2 (each 1C, s, 6,7,8, 9, 10, 11, 12-CH_2), 56.2 (1C, s, -OCH_3), 100.2 (1C, s, 4-CH_2), 109.3 (1C, s, 2'-CH), 114.9 (1C, s, 5'-CH), 120.2 (1C, 1, 2-CH), 122.4 (1C, 1, 6'-CH), 127.6 (1C, s, 1'-C), 139.8 (1C, 1, 1-CH), 146.5 (1C, s, 4'-C), 147.5 (1C, s, 3'C), 177 (1C, s, 3-COH), 202 (1C, s, C=O). MS ES+, 5.3·e^5: m/z: 347 (+H^+), 369 (+Na^+); ES-, 4.1·e^4: m/z: 345 (-H^+), 385.

6-Dihydroparadol:



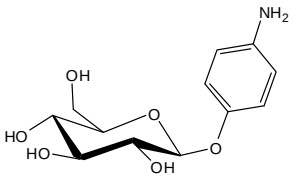
White crystals. > 95 % pure in HPLC. ^{1}H-NMR, 400 MHz, $CDCl_3$: δ = 0.9 (3H, t, 10-CH_3), 1.21-1.4 (10, m, $(CH_2)_5$), 1.4-1.53 (2H, m, 4-CH_2), 1.76-1.83 (2H, m, 2-CH_2), 2.59-2.78 (2H, m, 1-CH_2), 3.64 (1H, m, 3-C<u>H</u>OH), 3.9 (3H, s, -OCH_3), 5.49 (1H, s, arOH), 6.71 (1H, s, 6'-CH), 6.73 (1H, s, 2'-CH), 6.85 (1H, d, 5'-CH). ^{13}C-NMR, 400 MHz, $CDCl_3$: δ = 14.1 (1C, s, 10-CH_3), 22.6 (1C, s, 9-CH_2), 25.6 (1C, s, 5-CH_2), 29.3, 29.6, 31.8 (each 1C, s, 6,7,8-CH_2), 37.6 (1C, s, 4-CH_2), 39.4 (1C, s, 2-CH_2), 55.9 (1C, s, OCH_3), ~65 (1-CH_2), 71.5 (1C, s, -CHOH), 111 (1C, 1, 2'-CH), 114.2 (1C, 1, 5'-CH), 120.9 (1C, 1, 6'-CH), 134.1 (1C, 1, 1'-C), 143.7 (1C, 1, 4'-C), 146.4 (1C, 1, 3'-C). MS ES+, 4.9·e^5: m/z: 263 (-H_2O), 282 (+2H^+), 303 (+Na^+). IR: 3373, 2928, 2856, 2359, 2338, 1515 cm^{-1}.

Nitrophenyl glucose:



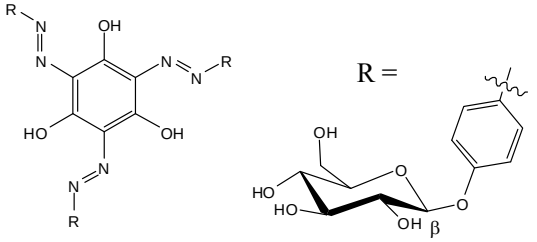
^{1}H-NMR, 400 MHz, methanol-d_4: δ = 3.3-3.5 (4H, m, -CH, -CH_2), 3.62 (1H, dd, -CH), 3.8 (1H, d, -CH), 5 (1H, d, β-CH), 7.15 (2H, t, 2',6'-arCH), 8.15 (2H, d, 3',5'-arCH). MS ES+, 1.9·e^6: m/z: 295, 307, 317.

Aminophenyl glucose:



^{1}H-NMR, 400 MHz, methanol-d_4: δ = 3.26-3.4 (4H, m, -CH, -CH_2), 3.62 (1H, dd, -CH), 3.8 (1H, d, -CH), 4.65 (1H, d, β-CH), 6.61 (2H, d, 3',5'-arCH), 6.86 (2H, t, 2',6'-arCH). MS ES+, 3.2·e^6: m/z: 272 (+H^+).

Yariv's Reagent:



^{1}H-NMR, 400 MHz, DMSO-d_6: δ = 3.2-3.4 (12H, t, -CH, -CH_2), 3.45 and 3.72 (6H, dm, CH_2), 4.6 (3H, t, COH), 4.93 (3H, d, β-CH), 5.04 (3H, d, 4-COH), 5.01 (3H, d, 2-COH), 5.35 (3H, d, 3-COH), 7.17 (6H, d, 2',6'-arCH), 7.65 (6H, broad s, 3',5'-arCH), 15.9 (3H, broad s, arOH). ^{13}C-NMR, 400 MHz, DMSO-d_6: δ = 60.7 (3C, s, CH_2), 70 (3C, s, 4-CH), 73.5 (3C, s, 2-CH), 76.6 (3C, s, -CH), 77 (3C, s, -CH), 100 (3C, s, β-CH), 117.6 (6C, s, 2',6'-arCH), 118.7 (6C, s, 3',5'-arCH), 128 (3C, s, arC), 128.5 (3C, s, arC).

Mosher's ester of 6-gingerol (crude): ^{1}H-NMR, 400 MHz, $CDCl_3$: δ = 0.82 (3H, t, 10-CH_3), 1.1-1.3 (8H, m, $(CH_2)_4$), 2.5-2.9 (6H, m, -CH_2-CH_2-CO-CH_2) 3.48 (2.7H, s, any 5-Mo-OCH_3), 3.72 (1.8H, s, any 4'-Mo-OCH_3), 3.79 (1.7H, s, 4'5-Mo_2-PhOCH_3), 3.81 (0.10H, s, 4'-Mo-PhOCH_3), 3.87 (1.15H, s, 5-Mo-PhOCH_3), 3.89 (0.05H, s, PhOCH_3), 5.49 (1H, m, C<u>H</u>O-Mo), 5.56 (0.35H, s, PhOH), 6.58-6.84 (2.25H, m, vanillyl), 6.84-6.91 (0.75H, d, 4',5-Mo_2-vanillyl, 5'-CH), 7.32-7.5 (6.65H, m, Mo-phenyl), 7.72 (1.25H, m, 4',5-Mo_2-phenyl, ortho-CH). MS ES+, 4.1·e^6: m/z: 240 (Mo +Na^+), 277 (6-S +H^+), 529 (6-G-Mo +H_3O^+), 530 (6-G-Mo +H^+ +H_3O^+), 534 (6-G-Mo +H^+ +Na^+), 745 (6-G-Mo_2 +H_3O^+), 746 (6-G-Mo_2 +H^+ +H_3O^+), 750 (6-G-Mo_2 +H^+ +Na^+).

-Mo = Mosher's ester, 5-Mo-X = alcohol adduct, 4',5-Mo_2-X = disubstituted product, 4'-Mo-X = phenyl adduct, PhOH = phenol of 6-gingerol, PhOCH_3 = phenyl of 6-gingerol, vanillyl = CH of the vanillyl moieties of any 6-gingerol derivative, 4',5-Mo_2-vanillyl, 5'-CH = 5'-CH of the vanillyl moiety of the Mosher's diester, Mo-phenyl = CH of the phenyl moieties of any Mosher's ester derivative, 4',5-Mo_2-phenyl, ortho-CH = ortho-CH of the phenyl moieties of the disubstituted Mosher's ester.

Peaks at 3.81 = 0.1 (4'-Mo-PhOCH_3) and 3.89 = 0.05 (6-gingerol), no peak at 4.02 (C<u>H</u>OH), peak at 5.49 = 0.9 (C<u>H</u>O-Mo), and peak at 5.56 = 0.35 (PhOH) → 6-gingerol ~2 %, desired alcohol-substituted product ~38 %, disubstituted product ~56 %, only phenyl adduct ~4 %. Nearly no visible shift for the peak at 3.48 (5-Mo-OCH_3): 6-Gingerol is > 95 % enantiopure.

Mosher's ester of 6-gingerol (dried and stored): ^{1}H-NMR, 400 MHz, $CDCl_3$: δ = 0.82 and 0.88 (3.5H, two t, 10-CH_3), 1.1-1.35 (8H, m, $(CH_2)_4$), 2.4-2.9 (6H, m, -CH_2-CH_2-CO-CH_2), 3.48 (2.02H, s, any 5-Mo-OCH_3), 3.72 (1.76H, s, any 4'-Mo-OCH_3), 3.79 (1.26H, s, 4',5-Mo_2-$PhOCH_3$), 3.81 (0.48H, s, 4'-Mo-$PhOCH_3$), 3.87 (0.95H, s, 5-Mo-$PhOCH_3$), 3.89 (0.35H, s, $PhOCH_3$), 4.02 (0.27H, m, C<u>H</u>OH), 5.42-5.53 (1.05H, m, PhOH and C<u>H</u>O-Mo), 6.58-6.92 (3H, m, vanillyl), 7.34-7.55 (5.32H, m, Mo-phenyl), 7.72 (1.19H, m, 4',5-Mo_2-phenyl). MS ES+, 4.1·e^6: m/z: 240 (Mo +Na^+), 277 (6-shogaol +H^+), 529 (6-G-Mo +H_3O^+), 530 (6-G-Mo +H^+ +H_3O^+), 534 (6-G-Mo +H^+ +Na^+), 745 (6-G-Mo_2 +H_3O^+), 746 (6-G-Mo_2 +H^+ +H_3O^+), 750 (6-G-Mo_2 +H^+ +Na^+).
Peak at 0.88 (-CH_3), peaks at 3.81 = 0.48 (4'-Mo-$PhOCH_3$) and 3.89 = 0.35 (6-gingerol), peak at 4.02 = 0.27 (C<u>H</u>OH), several shifts in the aromatic region → 27 % loss of the alcoholic Mosher's ester resulting in 6-gingerol 11.5 %, desired alcohol-substituted product ~31.3 %, disubstituted product ~41.4 %, only phenyl adduct ~15.8 %.

6-Gingerol-4'-Mosher's ester, LC fraction 2: ^{1}H-NMR, 400 MHz, $CDCl_3$: δ = 0.88 (3H, t, 10-CH_3), 1.2-1.35 (8H, m, $(CH_2)_4$), 2.4-2.95 (6H, m, -CH_2-CH_2-CO-CH_2), 3.72 (3H, s, 4'-Mo_2-OCH_3), 3.81 (3H, s, 4'-Mo-$PhOCH_3$), 4.02 (1H, m, C<u>H</u>OH), 6.73 (1H, d, 6'-CH vanillyl), 6.8 (1H, s, 2'-CH vanillyl), 6.9 (1H, d, 5'-CH vanillyl), 7.34 (3H, m, meta/para-CH 4'-Mo-phenyl), 7.72 (2H, m, ortho-CH 4'-Mo-phenyl). MS ES+, 7.6·e^6: m/z: 511 (6-G-Mo +H^+), 529 (6-G-Mo +H_3O^+), 535 (6-G-Mo +2H^+ +Na^+).

6-Gingerol-5-Mosher's ester, LC fraction 3: ^{1}H-NMR, 400 MHz, $CDCl_3$: δ = 0.82 and 0.88 (3H, two t, 10-CH_3), 1.2-1.35 (7.37H, m, $(CH_2)_X$), 1.43 (m, 6-shogaol 7-CH_2), 2.19 (0.89H, m, 6-shogaol 6-CH_2), 2.5-2.9 (4.74H, m, -CH_2-CH_2-CO- and -CH_2-CH_2-CO-CH_2), 3.48 (1.41H, s, 5-Mo-OCH_3), 3.54 (0.51H, s, isomer of 5-Mo-OCH_3), 3.87 (1.62H, s, 5-Mo-$PhOCH_3$), 3.89 (1.38H, s, 6-shogaol $PhOCH_3$), 5.45 (1.33H, m, PhOH and C<u>H</u>O-Mo), 6.09 (0.44H, d, 6-shogaol 4-CH), 6.6-6.85 (3H, m, CH vanillyl and 6-shogaol 5-CH), 7.34-7.6 (3.58H, m, CH 5-Mo-phenyl). MS ES+, 5.8·e^6: m/z: 267, 277 (6-shogaol +H^+), 299 (6-shogaol +Na^+), 511 (6-G-Mo +H^+), 529 (6-G-Mo +H_3O^+), 535 (6-G-Mo +2H^+ +Na^+).
~40 % desired product (S)-6-gingerol-5-Mosher's ester, ~45 % 6-shogaol, ~15 % (R)-6-gingerol-5-Mosher's ester (→ peak at 3.54!).

6-Gingerol-4',5-Mosher's diester, LC fraction 4: ^{1}H-NMR, 400 MHz, $CDCl_3$: δ = 0.82 and 0.88 (3H, two t, 10-CH_3), 1.15-1.35 (7.04H, m, $(CH_2)_X$), 1.43 (m, 6-shogaol 7-CH_2), 2.19 (0.88H, m, 6-shogaol 6-CH_2), 2.5-3 (5.04H, m, -CH_2-CH_2-CO- and -CH_2-CH_2-CO-CH_2), 3.48 (1.63H, s, 5-Mo-OCH_3), 3.54 (0.14H, s, isomer of 5-Mo-OCH_3), 3.72 (3H, s, any 4'-Mo-OCH_3), 3.87 (1.6H, s, 5-Mo-PhOCH_3), 3.89 (1.41H, s, 6-shogaol PhOCH_3), 5.48 (0.52H, m, C<u>H</u>O-Mo), 6.09 (0.43H, d, 6-shogaol 4-CH), 6.65-6.9 (3.42H, m, CH vanillyl and 6-shogaol 5-CH), 7.34-7.6 (6.18H, m, CH any 4'-Mo-phenyl), 7.72 (2.02H, m, any 4'-Mo-phenyl, ortho-CH). MS ES+, 4.5·e^{6}: m/z: 511 (6-G-Mo +H^+ or 6-S-Mo +H_3O^+), 516 (6-S-Mo +H^+ +Na^+), 745 (6-G-Mo_2 + H_3O^+), 750 (6-G-Mo_2 +Na^+), 751 (6-G-Mo_2 +H^+ +Na^+). ~54 % diester of (S)-6-gingerol, ~44 % 6-shogaol-4'-Mosher's ester, ~2 % diester of (R)-6-gingerol (→ peak at 3.54!).

3.14 Statistics

Results are expressed as mean values ± SD or SEM (depending on the experiment) for each examined group. Statistical significance between groups was determined with the Student's t-test (paired or unpaired) with GraphPad Prism software. Outliners in a series of identical experiments were determined by Grubb's test (ESD method) with a set to 0.05. Statistical differences between treated and vehicle control groups were determined by Student's t-test for dependent samples. Differences between the analyzed samples were considered as significant if $p \leq 0.05$, except for whole blood assays with $p \leq 0.1$.

K_i values were calculated using the Cheng-Prussof equation [333] based on Hill plots as reported previously [321].

4. Results and Discussion

4.1 Whole Blood Assay Setup

A high-content *in vitro* assay was established to profile single- and multi-component agents (e.g. pharmaceutical and botanical drugs) for their effectiveness as anti-inflammatory agents. In a 96-well setup, fresh human whole blood stimulated with a variety of pro-inflammatory compounds (Table 3) induced reproducible cytokine expression patterns, reflecting upon the activated cell populations and signal transduction pathways triggered. 18 h incubation was chosen based on experimental data on the *in vitro* kinetics of the cytokines of interest [277].

LPS, a CD14- and a MyD88- dependent TLR4 ligand [349], as expected induced monokines with strong TNF-α, IL-1β, IL-6, IL-8, and IL-10 induction (> 10'000 pg/ml, Fig. 11) [275]. The other cytokines (IL-12, GM-CSF, IL-3, IL-4, IL-5, and IL-7) measured were not or only weakly induced. On the other hand, combinations of the PKC-activating PMA [350] with the activating TCR-antibodies anti-CD3 (αCD3) and anti-CD28 (αCD28) [351, 352], and with the calcium ionophore thapsigargin [353] also induced T_H1 and T_H2 cytokines (GM-CSF, IL-3, IL-4, and IL-5), but less pronounced IL-1β, IL-6, and IL-10 expressions (Fig. 11). We defined a 'good' cytokine induction as an approximate 1'000-fold increase over baseline level for two reasons. First, to circumvent the high inter- and intra-individual variability (Fig. 11) and second, to avoid weak and possibly irrelevant effects. This necessitated the use of high concentrations of stimuli and resulted in only a few useful ones. Overall, this setup enabled us to detect reproducible and robust effects.

In order to validate this assay we next applied specific inhibitors of key inflammatory processes or signal transduction events, both clinically and experimentally used ones (Table 4). Interestingly, many of the highly-specific inhibitors often failed to exert a strong inhibition, whereas the corticosteroid dexamethasone strongly inhibited a broad set of cytokines and, as expected, the "calcineurin-antagonist" cyclosporin A inhibited mainly the calcium and TCR mediated T_H1 and T_H2 cytokines [306].

Coherent results for the two MEK inhibitors U0126 and PD 098059 and for the two p38 inhibitors SB 202190 and SB 203580, respectively, were lacking for most cytokines. To ensure selectivity, these inhibitors have been tested only slightly over reported IC_{50} values (Table 4) [354, 355]. Noteworthy, p38α is dispensable for mouse T and B cell function [356], behaves different in mouse and man [357], and is important for the suppressor function of iT_{reg} [250, 251] which might explain the up-regulation of GM-CSF, IL-4, IL-5,

and IL-7 in PMA/thapsigargin, PMA/αCD28, LPS, and ATP/thapsigargin stimulated whole blood. Nevertheless, all MEK and p38 inhibitors significantly inhibited LPS-stimulated IL-1β expression but with a high inter-assay variability. On the other hand, the COX-2 inhibitor diclofenac and the H1-antagonist cetirizine tendentially enhanced the induction of most cytokines independent of the stimulus and the sPLA2 inhibitor DBA exerted no effects. Since whole-blood from different donors (independent of gender and age) was employed we concluded that the effects observed were robust and meaningful.

Stimulus	Concentration	Effect alone	Comments
αCD3 Σ-chain	1 μg/ml	poor	→ combined with PMA
αCD28	2 μg/ml	poor	→ combined with PMA
Arachidonic acid	10 μg/ml	none	→ assayed in combination
ATP	10 μM	none	→ used in combination
Calcitriol	10 nM		→ assayed in combination
Candida albicans	10^4/ml	good	→ too similar to LPS
fMLP	100 nM	poor	→ assayed in combination
Ephedrin	10 μM		→ assayed in combination
Formaldehyde	100 μg/ml	none	→ not further used
Forskolin	25 μM	moderate	→ assayed in combination
H_2O_2	500 μM	none	→ not further used
LPS	312.5 ng/ml	strong	→ strong monokine induction
Lymphocytes	10^4/ml	poor	→ not further used
Nickel chloride	300 μM	none	→ not further used
PHA	2 μg/ml	poor	→ not further used
PMA	15 ng/ml	moderate	→ used in combination
ATRA	10 nM		→ assayed in combination
Thapsigargin	1 μM	moderate	→ used in combination
TNF-α	100 ng/ml	moderate	→ not further used

Table 3: Stimuli tested in whole blood.

Anti-TCR antibodies and LPS were chosen due to their well established effects and therefore their optimal concentrations were titrated. Antibodies were combined with a low dose of PMA to elicit full T cell activation. Concentrations for the other stimuli are based on literature data. Several stimuli were used in combination but, besides combinations with PMA, usually resulted in a weak cytokine induction, and therefore were not further used.

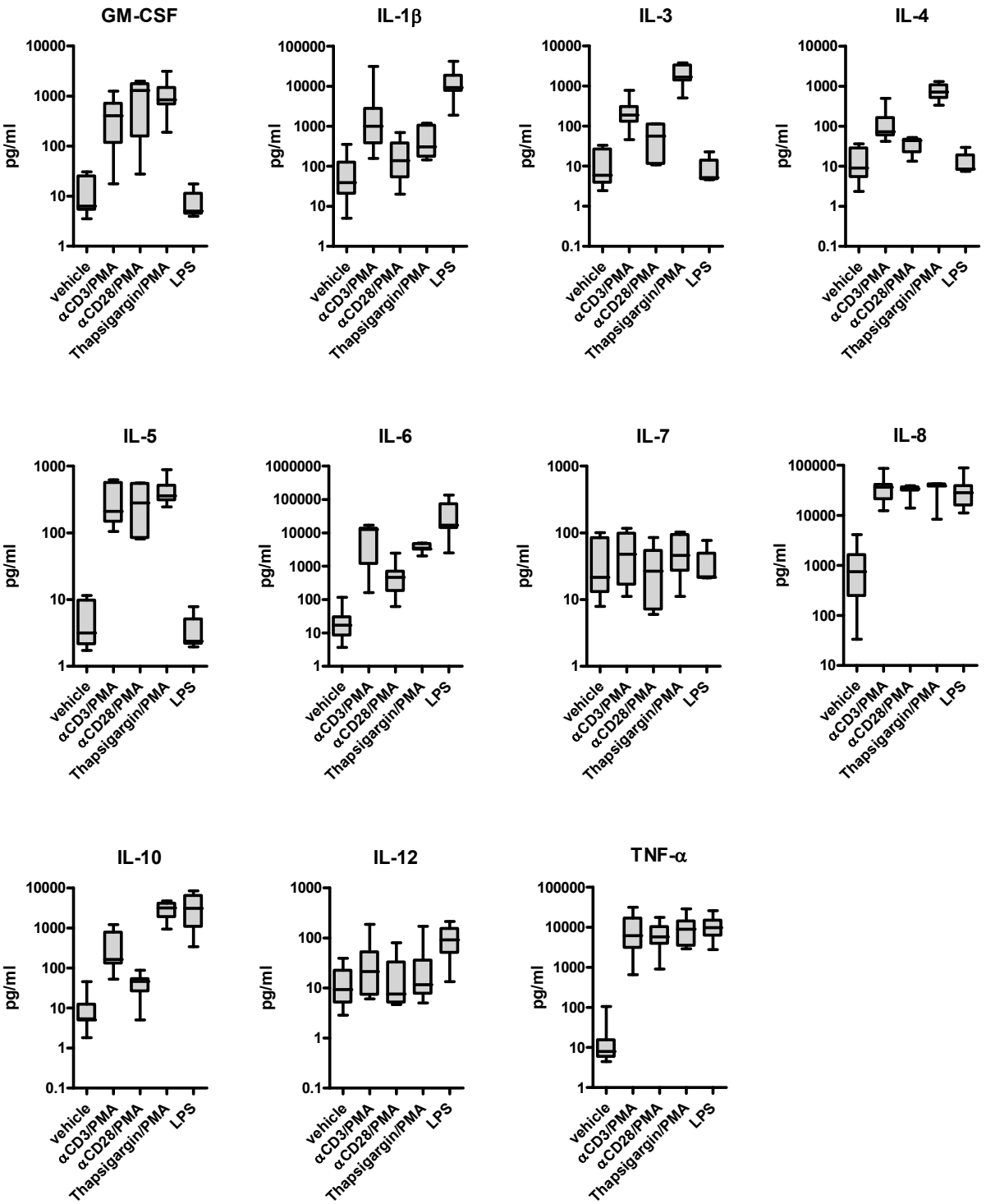


Figure 11: Stimulated cytokine expression in whole blood.

Shown are box-and-whisker plots of expression levels (y-axis) of all measured cytokines in differential stimulated (x-axis) human whole blood. Clearly visible are the high variability in the expression levels under constitutive (vehicle) as well as stimulated conditions (three combinations of PMA and LPS, respectively) after 18 h incubation. The results of 4 to 35 experiments involving at least 4 different donors are given.

Inhibitor	Concentration	Comment
Cetirizin	350 ng/ml	≈ C_{max} of a 10 mg dose
Cyclosporin A	800 ng/ml [278]	≈ C_{max} at steady state at 100 mg/d
DBA	10 μM	Commonly used concentration
Dexamethason	100 nM [358]	A 5 mg dose corresponds to ≈ 80 ng/ml of free compound in plasma
Diclofenac	1 μg/ml	C_{max} ≈ 0.5-1.5 μg/ml single dose
Parthenolide	5 μM	Commonly used concentration
PD 098059	10 μM	IC_{50} MEK1: 2-7 μM [354, 359]
SB 202190	100 nM	IC_{50} SAPK2a/p38: 50 nM IC_{50} SAPK2b/p38b2: 100 nM [354, 360]
SB 203580	50 nM	IC_{50} SAPK2a/p38: 50 nM IC_{50} SAPK2b/p38b2: 500 nM [354, 361]
SP600125	100 nM	IC_{50} JNK1, 2, 3: 40-90 nM [355, 362]
U0126	100 nM	IC_{50} MEK1: 72 nM IC_{50} MEK2: 58 nM [354, 363]
DPAT	100 nM 100 μM	Two concentrations chosen due to unknown endogenous 5-HT levels
NAN-190	100 nM 100 μM	Two concentrations chosen due to unknown endogenous 5-HT levels

Table 4: Concentrations of tested inhibitors and modulators.

The inhibitors were chosen because i) they are approved drugs for the treatment of inflammation, allergy, and/or organ rejection (maximal plasma concentrations are indicated and used in the assay), ii) are well established *in vitro* inhibitors of MAP kinases (concentrations around the IC_{50} values were used in the assay), NF-κB, or $sPLA_2$, or iii) are ligands of the 5-HT_{1A}R subtype.

Whole blood showed a high threshold for stimuli-induced cytokine expression and for drug induced inhibition as well. This may be explained by the following facts: i) the ability of immune cells to become anergic upon partial stimulation [364], ii) interference with counter-acting feed-back loops (e.g. diclofenac reduces PGE_2-mediated inhibition [365]), and/or iii) the activity of several parallel pathways compensating for the one inhibited. On the other hand, a truly significant effect can then be assumed to be strong and might therefore be of physiological relevance (e.g. dexamethasone and cyclosporin A).

4.2 Immunopharmacological Profiling of Ginger Extracts

4.2.1 Whole Blood

Based on our assumption that certain ginger constituents may modulate the expression of pro-inflammatory cytokines, distinct ginger extracts were analysed (Table 5). Additionally, other anti-inflammatory medical plants, like *Harpagophytum procumbens*, *Salix sp.*, and curcumin were tested in the same setup (Table 5).

The ginger Hot Flavor™ extract was able to inhibit the expression of several cytokines in stimulated whole blood at 50 μg/m. Mainly PMA/αCD3 induced cytokines (TNF-α, IL-6, and IL-10) and IL-1β for all stimuli were inhibited. IL-1β, induced by PMA combinations, was also inhibited by the ginger CO_2 totum extract. Furthermore, both CO_2 extracts inhibited PMA/αCD3 stimulated IL-6 and augmented PMA/thapsigargin induced TNF-α. Because the ginger CO_2 totum extract exerted a less pronounced inhibition pattern than the ginger Hot Flavor™ extract (which is partially deprived of essential oil), the latter one was used for all further assays. The stimulatory effect of the hydro-alcoholic ginger extracts (V61101 and V61501) on several PMA/αCD28 and ATP/thapsigargin induced cytokines (Table 5) provided the basis for the AGP part of this work (see chapter 4.5).

Notably, the concentration of 50 μg/ml ginger Hot Flavor™ extract corresponds to a total gingerol/shogaol content of 66 μM and a 6-gingerol concentration of 42 μM. Therefore pure ginger constituents were tested at 50 μM. But unlike the ginger Hot Flavor™ extract, 6-, 8-, 10-G, and 6-S only weakly inhibited the cytokine expression with the exception of PMA/αCD28 triggered IL-6 and IL-10 and PMA/thapsigargin triggered IL-1β and IL-10 which were inhibited with up to 70%.

Curcumin (a mixture of curcumin, desmethoxy-, and bisdesmethoxycurcumin) at 25 μg/ml gave a similar inhibition pattern as the ginger Hot Flavor™ extract but with a marked inhibition of IL-10 for all stimuli used. On the other hand, *Harpagophytum procumbens* extracts (50 μg/ml), only tested in PMA/αCD3 and LPS stimulated whole blood, exerted no effects and the *Salix sp.* extract (50 μg/ml) only caused minor alterations of stimulated cytokine expression (Table 5).

Ginger Hot Flavor™	TNF-α	IL-6	IL-1β	IL-8	IL-10
PMA/αCD3	72 ± 5**	61 ± 6***	38 ± 3***	99 ± 2	65 ± 7**
PMA/αCD28	113 ± 11	39 ± 3***	64 ± 4***	98 ± 2	86 ± 8
PMA/thapsigargin	123 ± 3***	103 ± 2	52 ± 13	101 ± 1	103 ± 4
ATP/thapsigargin	102 ± 13	91 ± 11	98 ± 1*	148 ± 34	100 ± 0
LPS	98 ± 5***	100 ± 0	63 ± 4**	119 ± 3	99 ± 3

Ginger CO_2 totum	TNF-α	IL-6	IL-1β	IL-8	IL-10
PMA/αCD3	100 ± 1	100 ± 0	73 ± 3**	102 ± 1	86 ± 6
PMA/αCD28	98 ± 1	56 ± 6**	58 ± 6**	103 ± 0**	84 ± 1**
PMA/thapsigargin	121 ± 5*	99 ± 6	36 ± 5**	105 ± 0**	85 ± 1***
LPS	122 ± 2**	100 ± 0	97 ± 6	106 ± 1*	110 ± 2*

Ginger V61101	TNF-α	IL-6	IL-1β	IL-8	IL-10
PMA/αCD3	90 ± 6	108 ± 4	82 ± 2**	104 ± 0***	108 ± 10
PMA/αCD28	143 ± 5**	624 ± 169	364 ± 43**	94 ± 3	166 ± 13*
PMA/thapsigargin	125 ± 10	113 ± 7	187 ± 43	100 ± 1	101 ± 3
ATP/thapsigargin	220 ± 68	2086 ± 698	195 ± 41	110 ± 15	272 ± 75
LPS	75 ± 6*	100 ± 0	69 ± 6*	105 ± 2	104 ± 3

Ginger V61501	TNF-α	IL-6	IL-1β	IL-8	IL-10
PMA/αCD3	101 ± 8	108 ± 3	71 ± 5*	103 ± 0***	98 ± 12
PMA/αCD28	139 ± 5**	577 ± 185	236 ± 34*	100 ± 0	133 ± 12
PMA/thapsigargin	123 ± 10	108 ± 6	254 ± 72	103 ± 1	99 ± 5
ATP/thapsigargin	209 ± 84	1946 ± 804	102 ± 1	103 ± 5	123 ± 19
LPS	68 ± 8*	100 ± 0	55 ± 8*	102 ± 3	85 ± 8

Curcumin	TNF-α	IL-6	IL-1β	IL-8	IL-10
PMA/αCD3	76 ± 6*	69 ± 4**	30 ± 5***	87 ± 6	31 ± 3***
PMA/αCD28	80 ± 3**	69 ± 2***	91 ± 6	104 ± 1*	40 ± 4***
PMA/thapsigargin	96 ± 8	146 ± 23	65 ± 7*	104 ± 1*	34 ± 3***
LPS	138 ± 8*	105 ± 1	89 ± 2*	219 ± 18**	63 ± 7*

Salix sp.	TNF-α	IL-6	IL-1β	IL-8	IL-10
PMA/αCD3	103 ± 3	92 ± 2	97 ± 11	94 ± 5	69 ± 3**
LPS	94 ± 2	118 ± 1***	87 ± 4	178 ± 4***	106 ± 1*

H. procumbens 1	TNF-α	IL-6	IL-1β	IL-8	IL-10
PMA/αCD3	93 ± 2	97 ± 3	86 ± 7	97 ± 3	89 ± 1**
LPS	102 ± 6	97 ± 2	90 ± 3	101 ± 3	96 ± 1

H. procumbens 2	TNF-α	IL-6	IL-1β	IL-8	IL-10
PMA/αCD3	94 ± 2*	94 ± 4	92 ± 13	90 ± 7	89 ± 4
LPS	110 ± 10	103 ± 1	102 ± 5	93 ± 4	97 ± 0*

Table 5: Cytokine modulation in stimulated whole blood by plant extracts.

Fresh human whole blood was stimulated with 50 µg/ml of plant extracts, incubated for 30 min at 37°C and indicated pro-inflammatory stimuli were added. Cytokines were measured with CBA and FACS after an incubation of 18 h at 37 °C.

Shown are mean values ± SEM as percent of the corresponding vehicle controls. Blood samples from at least three different donors were used and each measured in a triplicate. * $P \leq 0.1$; ** $P \leq 0.05$; *** $P \leq 0.01$.

As shown in Table 5, the ginger Hot Flavor™ extract potently inhibited IL-1β expression (≥ 30 %) for all stimuli significantly inducing this cytokine (Fig. 11). All other cytokines were less or not inhibited, which led us to the conclusion that ginger constituents are able to selectively and globally interfere with the IL-1β expression machinery by concrete mechanisms. Since the different stimuli induce distinctly different signal transduction events we excluded the possibility that upstream events, such as e.g. inhibition of MAP kinases or transcription factors, are responsible for this effect. Because PLA_2 enzymes have been shown to be crucial for efficient IL-1β expression in monocytes/MΦ [142] we speculated that ginger constituents may inhibit PLA_2.

4.2.2 Targeting IL-1β and Phospholipase A_2 Pathways

IL-1β was the only cytokine in whole blood which was inhibited robustly by ginger extracts essentially independent of the stimulus used (Fig. 12 A and B). Therefore a standard IL-1β assay with isolated monocytes was performed to reproduce this effect with those cells that most likely release this interleukin.

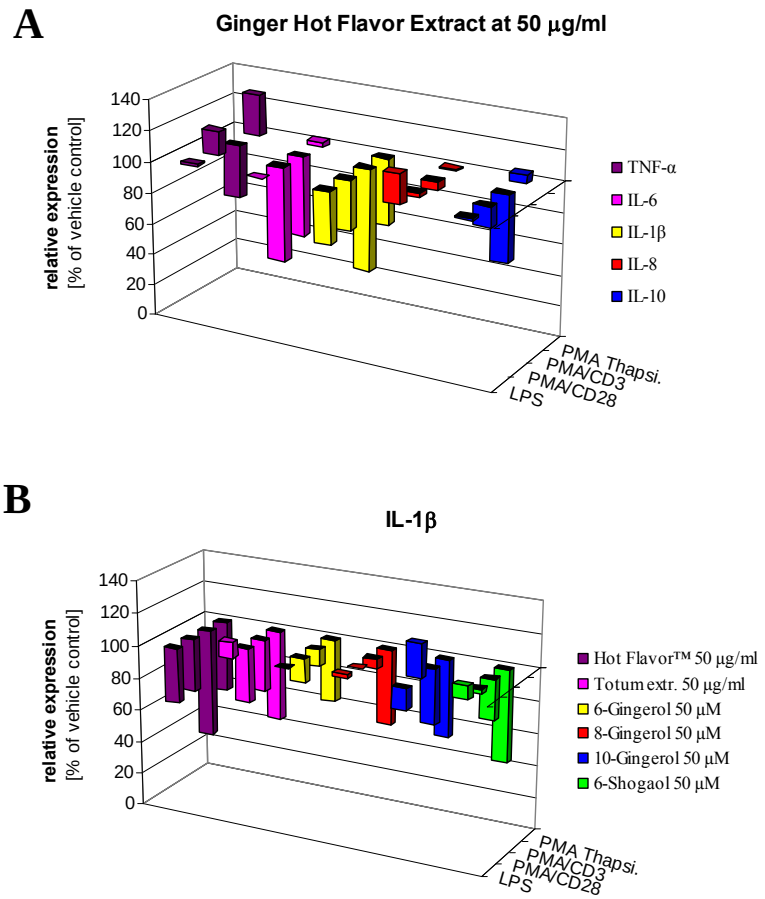


Figure 12: Modulation of the cytokine expression in whole blood by ginger.

A Modulation pattern of the ginger Hot Flavor™ extract on cytokine expression in stimulated whole blood. As the only cytokine, IL-1β is inhibited by at least 30 % with all indicated stimuli.

B Modulation pattern of two ginger CO_2 extracts and their main constituents on IL-1β expression in stimulated whole blood. The essential oil deprived ginger Hot Flavor™ extract was the only sample inhibiting IL-1β expression for all stimuli.

Shown are the mean values as relative expression compared to their vehicle controls. Blood of at least three different donors was used.

Isolated human monocytes secrete about 4 pg mature IL-1β per $2 \cdot 10^5$ cells within 4 h in culture and around 59 pg when incubated (primed) with LPS. These constitutive secretions are statistically unchanged by 10 µg/ml ginger extract (2 pg and 45 or 62 pg when added prior or after LPS, respectively); likewise, 1 mM ATP does not change cytokine secretion in non-primed cells (4 pg). On the contrary, stimulating primed cells with ATP causes a strong increase in cytokine secretion to around 183 pg.

This stimulated secretion was nearly completely abrogated (to 68 pg) when ginger extract was added prior to the LPS priming and dropped to 116 pg when added after LPS and prior to ATP (relative expression is shown in Fig. 13). These secretion levels are significantly different from LPS plus ATP but insignificantly so compared to LPS alone. The results demonstrated a specific inhibition of IL-1β expression and led to further experiments (described below) in which the molecular target was elucidated.

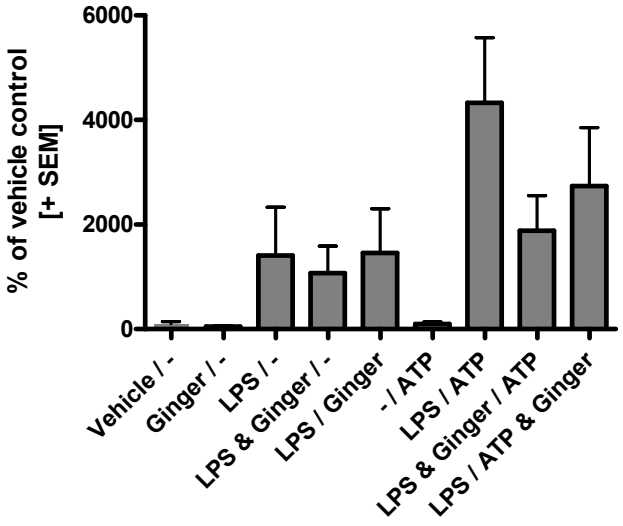


Figure 13: IL-1β secretion in LPS primed and ATP stimulated human monocytes.
Human monocytes were primed for 4 h (e.g. with vehicle control, LPS, Ginger or both) and afterwards 30' stimulated (e.g. with vehicle control, ATP, ginger or a combination of both). Ginger extract (10 μg/ml) was added 30' either before LPS or ATP. Shown is the relative expression (+SEM) of mature IL-1β in the culture medium compared to the vehicle control. $P \leq 0.05$ for LPS/- vs. vehicle, LPS/ATP vs. LPS/-, LPS and Ginger/ATP vs. LPS/ATP (but not vs. LPS/-), and LPS/ATP and Ginger vs. LPS/ATP (but not vs. LPS/-); n=4

Ethidium$^+$ uptake

Ethidium$^+$ uptake after ATP stimulation is regarded as equivalent to general cation influx through large $P2X_7$ receptor adjacent pores and was unchanged by ginger extract as measured by flow cytometry in isolated monocytes (Fig. 14). The time vs. fluorescence mean value plots (Fig. 14) were identical and an inhibition of either ATP binding to its receptor or fast ion fluxes (potassium out, calcium in) can be excluded.

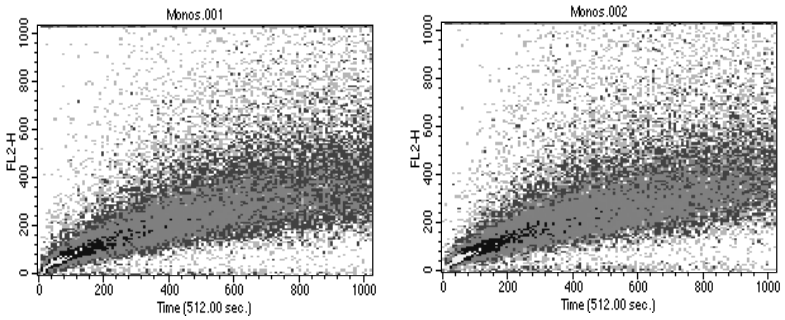


Figure 14: ATP-mediated ethidium$^+$ influx in isolated monocytes.

Representative graphs of the time resolved (x-axis) ethidium$^+$ fluorescence (y-axis) of vehicle control (left) and ginger extract at 10 µg/ml (right) in LPS primed and ATP stimulated monocytes (ATP added at 0 sec.).

Western Blot Analysis

Differentiation between intra- and extra-cellular pro- and mature IL-1β was possible on Western blot even though inhibition was less pronounced with the used 'gel electrophoresis friendly' assay conditions. In isolated human monocytes the $iPLA_2$ dependent maturation and the $cPLA_2$ dependent secretion are reduced to 63 % and 65 %, respectively, at 10 µg/ml ginger extract, whereas transcription/translation and constitutive maturation/secretion were unaffected (Fig. 15).

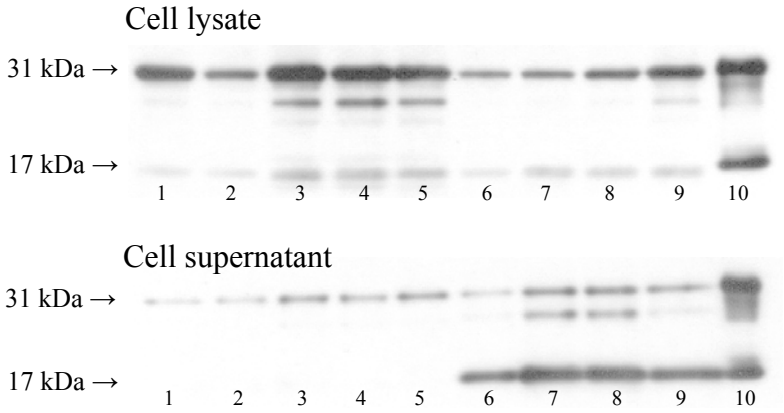


Figure 15: Western blot of pro- and mature IL-1β in isolated monocytes.

IL-1β was detected in cell lysates (upper) and in the incubation buffer (lower) after 60' stimulation of monocytes in gluconate BSS. 31 kDa: pro-IL-1β, 17 kDa: mature IL-1β. Line 1: vehicle control; 2-4: priming with ginger, LPS, and ginger prior to LPS; 5: LPS priming plus ginger stimulation, 6: only ATP stimulation, 7-9: LPS priming with ATP stimulation: alone, with ginger prior to LPS, and ginger prior to ATP, and 10: pro- and mature IL-1β standards.

Phospholipase assay

A new fluorescence coupled PLA_2 assay which has a low detection limit without the use of radioactive substrates was established to minimize required enzyme quantities and expensed. Furthermore, the assay is suitable for HPLC coupling, includes no critical handling step (e.g. sonication), and can be used to discriminate the three main PLA_2 groups. Notably, PLA_2 require surface-bound substrates for catalysis; several common assay principles using different substrates, lipid carriers, and detection methods are available [366, 367] but each one had some inconveniences for our requirements. The most common principles are listed below; the detection limits are an approximation from a variety of publications:

- Medium acidification by liberated fatty acids:
  - Titrimetric quantification (>50-100 nmol/min/ml)
  - pH indicators
- Radiochemical procedures (fmol to pmol detection limit) including liquid extraction of a substrate followed by TLC separation and quantification with a scintillation counter:
  - Dole-extraction of [^{3}H or ^{14}C] free fatty acid [368, 369]
  - Folch or Bligh & Dyer extraction of [^{3}H, ^{14}C or ^{32}P] lyso-PC
  - [^{135}I] BHC12-phosphatidylcholine
- Coupled enzyme assays (20-400 pmol/min/ml) [370, 371]
- Spectrophotometric thiol detection (>1-10 nmol/min/0.1ml)
  - Products of thioester or carbonothioate substrates coupled to DTNB or DTP [372, 373]
  - Fluorometric (>10 pmol/min/0.1ml)
- Fluorescent membrane probes, FRET and excimer-substrates [374-378]
- Acylcoumarine derivatives (e.g. umbelliferyl arachidonate) [379]
- Electron spin resonance spectroscopy (1 nmol/min/0.1ml)
  - Spin-labelled PC derivatives [380]

Optimal results were achieved by the use of 8 mM palmitoyl ascorbate potassium salt (PAK) as a lipid carrier containing 1 mM Tween 80 and 1 mM thio-PC as a substrate and 100 mM Good´s buffer adjusted to pH 7.4 at 40°C. Thio-PC exposes a free thiol after enzymatic hydrolysis and was coupled to monobromobimane (mBrB), a fluorescent sulfhydryl detection reagent (Fig. 16). The resulting adduct was quantified by fluorescence detector coupled HPLC.

PAK and Tween 80 were chosen as carriers in order to avoid Triton X-100 and sonication, while obtaining small negatively charged micelles (Fig. 17), self forming at >35 °C and concomitantly protecting disulfide formation [381]. The non-ionic surfactant Tween 80 enhances phospholipid solubilisation [382] and increased mixed micelle stability. Furthermore, polyethylenglycol containing lipid carriers are reported to facilitate binding of phospholipases to surfaces ($sPLA_2$) and/or enhance enzymatic activity ($iPLA_2$). Unsufficient hydrolysis and or stability was achieved by the following surfactants tested at 10 to 20 mol-% in PAK micelles: 1-stearoyl-2-arachidonoyl-sn-glycerol, Span-20, Span-80, distearoyl phosphatidic acid (DSPA), Tween-20, polydocanol, Triton X-301, Triton X-100, N-lauroylsarcosin, isolated phosphatidyl inositol, and sucrose monolaurate. MOPS buffer pH adjusted with KOH showed best results.

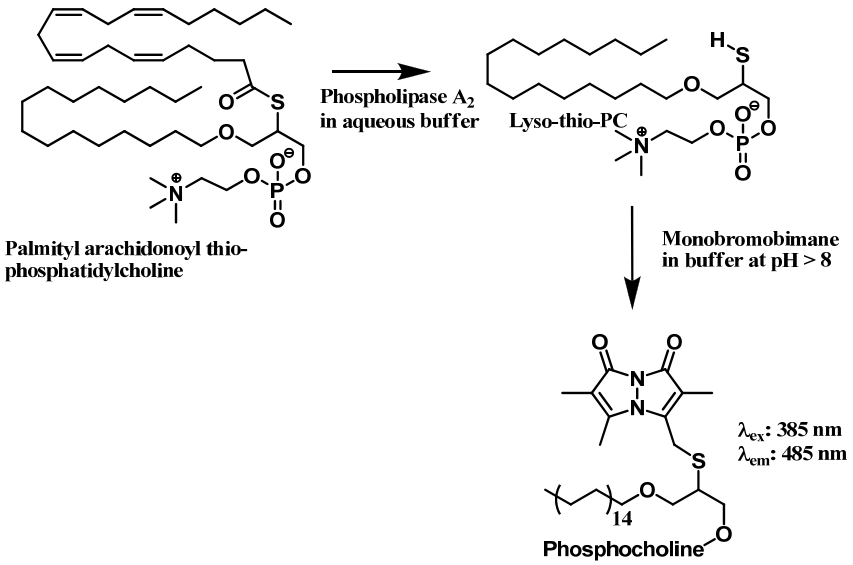


Figure 16: Fluorescence coupled PLA_2 assay.

The substrate palmityl arachidonoyl thio-phosphatidylcholine (thio-PC), incorporated into mixed micelles, was incubated with PLA_2 containing cell lysates at 40 °C for 3 h. The generated lyso-thio-PC derivative contains a free thiol which was coupled to mBrB under basic conditions. The reaction was quenched by acidification to alter the product stability. The highly fluorescent thioether adduct was quantified by HPLC with a fluorescence detector.

The enzymatic reaction was quenched with methanol and eventually formed dithiols reduced with DTT. Calcium, which interfered with the subsequent manipulations, was chelated with EDTA caesium salt to enhance the coupling reaction of the lyso-thio-PC

product with mBrB [342]. mBrB was used instead of the originally reported DTNB [372] because the resulting fluorescent adduct (structure see Fig. 16) has a several fold lower detection limit than the colored nitrobenzoic acid derivative. Strategies for the workup of the enzymatic reaction which were not successful were the Dole lipid extraction of lyso-thio-PC [383], reduction and protection of lyso-thio-PC and/or protein thiols with sodium dithionite or thiosulfate [384], and tetrabutylammonium iodide for enhanced bimane coupling as recommended by Salvatore et al. [342] (caused fast degradation of mBrB to bimane and its coupling to ascorbic acid).

Inhibition of phospholipases with standard inhibitors (MAFP, BEL) was only approximately 50 %. This might be due to one of the following reasons: First, Ca^{2+} and phospholipase independent hydrolytic activities. Second, Ca^{2+} and BEL insensitive PLA_2 activity as described by Balsinde [209]. Third, pre-incubation of the inhibitors as usually done when testing irreversible inhibitors was omitted [368, 369]. This was on purpose to ameliorate the comparability with the most likely non-covalent inhibition of ginger constituents embedded into the mixed micelles.

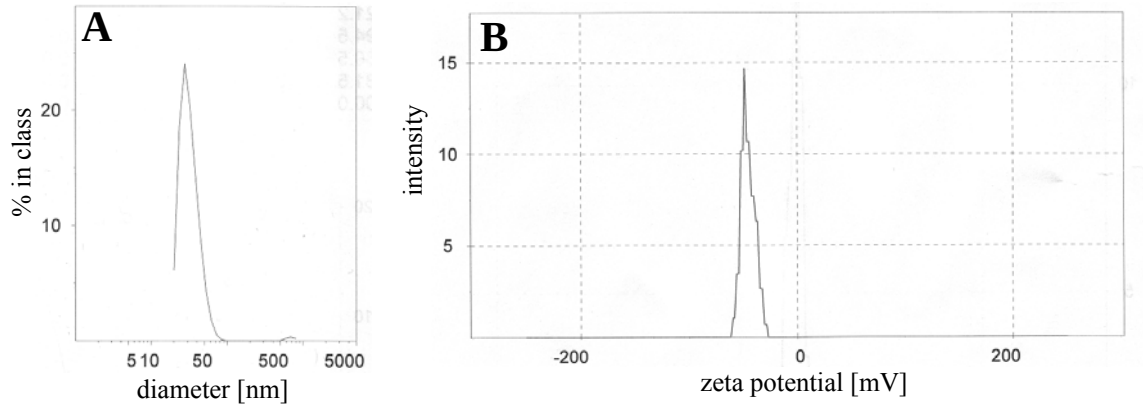


Figure 17: Size and zeta potential of the mixed micelles.

Shown are representative graphs of **A** the size (volume distribution) and **B** the zeta potential of mixed micelles (8 mM PAK, 1 mM Tween 80, 1 mM thio-PC) in incubation buffer (190 mM MOPS, 152 mM KOH, 3 mM DTT, 2 mM ATP, 1 mg/ml BSA) prepared as described in "Materials and Methods" analyzed on a ZetaSizer.

$cPLA_2$ activity was low; increasing the pH to 8, stimulating cells prior to lysis to induce enzyme phosphorylation, or addition of PIP_2 in the assay buffer could alter its activity [385]. Porcine $sPLA_2$ worked on the used mixed micelles too, maybe because the mM calcium concentration led to micelle fusion. Additionally, this enzyme showed an

approximately 100 times higher activity than expected, most likely due to the exceptionally negatively charged micelle surface.

The ginger Hot Flavor™ extract and its main constituents inhibited the activities of the i- and $cPLA_2$ isoforms isolated from U-937 cells in the same concentration range as the irreversible inhibitors MAFP [386-388] and BEL [389, 390].

Relative activity of both enzymes was reduced to ~50 % with 10 µg/ml extract and 10 µM pure compounds, respectively. For $iPLA_2$ the 8 homologues are somewhat less active and 10-gingerol and 6-shogaol inhibit stronger than the tested inhibitors with an overall inhibition of approximately 65% (Fig. 18). For $cPLA_2$ the shogaol series and the 10 homologues within each series show a tendency to be more potent than 6- and 8-gingerol, which inhibit $cPLA_2$ by approximately 30% (Fig. 19). 10-shogaol and the ginger Hot Flavor™ extract show a dose-dependent inhibition of both isoforms. The inhibition of $iPLA_2$ was stronger at lower concentrations compared to the inhibition of $cPLA_2$, in particular with the ginger extract (EC_{50} values 0.7 µg/ml versus 3 µg/ml). These findings have been published [328].

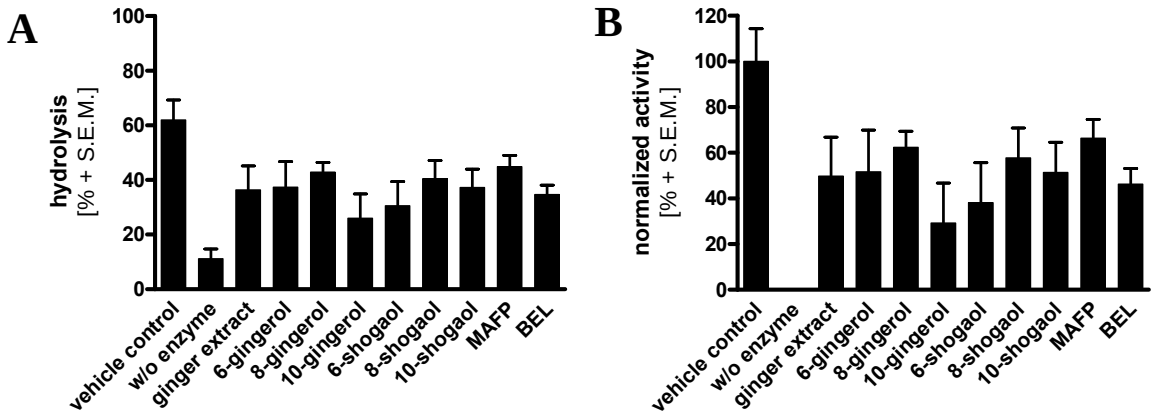


Figure 18: $iPLA_2$ activity on mixed micelles.

The ginger Hot Flavor™ extract (10 µg/ml), its main constituents (10 µM), and the two inhibitors MAFP and BEL (each 10 µM) were tested on mixed micelles for their inhibition of $iPLA_2$ isolated from U-937 cells. Shown are **A** the absolute hydrolysis expressed as amount of hydrolysed thio-PC (+ SEM) and **B** the normalized activity expressed as percent hydrolysed compound compared to the vehicle control (+ SEM) after 3 h incubation at 40 °C. $n \geq 3$

Thus, a structure-activity relationship was not visible as all gingerols and shogaols inhibited i/cPLA$_2$ enzymes. Additionally, inhibition caused by surface dilution of the micelles can be excluded because the concentration of inhibitors compared to the lipid carriers was too small and there is no correlation with their molecular sizes [391]. Porcine pancreatic sPLA$_2$ activity was tested using isolated phosphatidylcholine and an established TLC method [317, 318], was not inhibited by the ginger extract up to 20 µg/ml. In contrary, the nonspecific enzyme inhibitor DBA exerted a strong inhibition (data not shown).

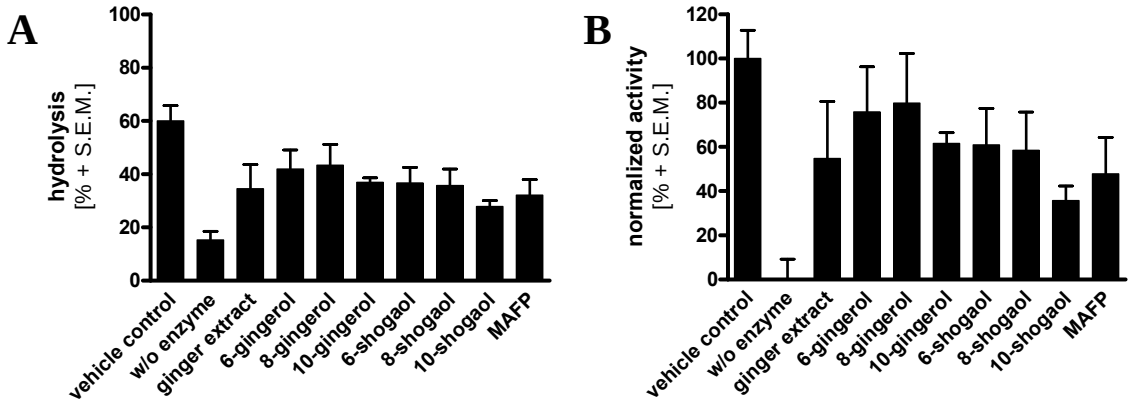


Figure 19: cPLA$_2$ activity on mixed micelles.

The ginger Hot Flavor™ extract (10 µg/ml), its main constituents (10 µM), and the inhibitor MAFP (10 µM) were tested on mixed micelles for their inhibition of cPLA$_2$ isolated from U-937 cells. Shown are **A** the absolute hydrolysis expressed as amount of hydrolysed thio-PC (+ SEM) and **B** the normalized activity expressed as percent hydrolysed compound compared to the vehicle control (+ SEM) after 3 h incubation at 40 °C. $n \geq 3$

PLA$_2$ inhibition in whole blood

During the early work, 2,4'-dibromoacetophenone (DBA), an irreversible sPLA$_2$ (and probably week cPLA$_2$) inhibitor, was tested in the whole blood assay. DBA inhibited LPS stimulated expression of TNF-α by 10 % and of IL-1β insignificantly by 20 %. Furthermore, a significant inhibition of PMA/αCD3 mediated expression of IL-3, IL-4, and IL-5 by 15 to 25 % was observed. All other stimuli and cytokines were not significantly modulated. These modulation patterns were completely different from the ones obtained with ginger preparations and are therefore in accordance with the lacking inhibition of sPLA$_2$ by ginger constituents. Obviously, it would have been interesting to also test the aforementioned strong i- and cPLA$_2$ inhibitors BEL and MAFP.

4.2.3 Inhibition of PEG_2 and Arachidonic Acid Release in U-937 Macrophages

Based on the measured inhibition of PLA_2 enzymes by ginger phenylpropanoids in the mixed-micelle assay (Fig. 18 and 19) we next assessed the effects of ginger constituents on free AA levels and PGE_2 release from U-937 macrophages. A quantitative GC-MS analysis was employed to determine both PGE_2 levels in cell supernatants (cell medium) and in the cellular fractions (see methods section). While AA could be detected in both supernatant (5.4 nmol/$1 \cdot 10^7$ cells) and in the thoroughly washed cellular fraction (0.4 nmol/$1 \cdot 10^7$ cells) of native U-937 cells, PGE_2 was only found in the supernatant of U-937 cells differentiated into macrophages. These cells constitutively released PGE_2 even without LPS stimulation (0.1 nmol/$1 \cdot 10^7$ cells). Upon stimulation by LPS, the PGE_2 levels stably increased by approximately 3-fold. As shown in Fig. 21, the positive control acetyl salicylate (Aspirin™) at concentrations at which COX-1/2 is inhibited *in vitro* (> 10 μM) dose-dependently inhibited PGE_2 release from LPS-stimulated U-937 cells. The PLA_2 and fatty acid amide hydrolase inhibitor MAFP (0.5 μM) and 10-shogaol (2 μM) inhibited PGE_2 and the prostanoid metabolite TXB_2 release by more than 50%. In non-stimulated macrophages, 10-S inhibited constitutive PGE_2 expression by more than 50% [328]. Low concentrations of ginger Hot Flavor™ extract (2 μg/ml) showed a significant inhibition of PGE_2 and TXB_2 release, whereas LTB_4 was only weakly inhibited. As expected, the $iPLA_2$ selective inhibitor BEL had no effect on PGE_2, TXB_2, and LTB_4 production, whereas the nonselective i-/$cPLA_2$ inhibitor MAFP also inhibited LTB_4 [328]. On the other hand, PEA, which is constitutively released by U-937 macrophages (0.1 nmol/$1 \cdot 10^7$ cells), was not modulated by ginger extract or 10-S. LPS did not increase PEA secretion. Somewhat unexpectedly, ginger extract and 10-S significantly increase the free AA levels in U-937 macrophages both in the cellular fraction and in the supernatant under both untreated and LPS stimulated conditions. MAFP reduced free AA only in the supernatant whilst, as expected, acetyl salicylate did not influence free AA levels.

The inhibition of prostaglandin E_2 expression in monocytes by ginger constituents is well established, for example by Jolad et al. [12, 16]. Also, $cPLA_2$ group IVA is linked to cyclooxygenases [392] and its inhibition decreases PGE_2 synthesis [253] and the main molecular targets of NSAID are cyclooxygenases (often equalised to PGE_2 synthesis) [393]. On the other hand, diclofenac, in the whole blood assay, led to a statistically insignificant enhanced cytokine production of 20 to 100% for most measured cytokines and most used stimuli (the modulation of PMA/αCD3 stimulation is shown as a representative example in

Fig. 20). This finding can be explained by the loss of suppressive effects mediated by G_s coupled E prostanoid receptors 2 and 4 [394], the same cAMP-dependent mechanism described for serotonin receptor mediated T cell inhibition. Additionally, inhibition of COX-1 derived PGE_2 is responsible for major side-effects on gastric mucosa caused by non-selective NSAID [393]. Therefore, reduced PG, and especially PGE_2, synthesis alone seems unlikely to be the dominant action of ginger *in vitro* as well as *in vivo*.

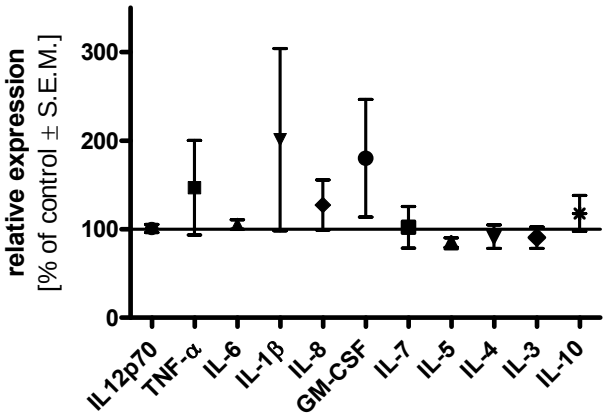


Figure 20: Cytokine expression pattern of diclofenac in stimulated whole blood.

Human whole blood, stimulated with PMA/αCD3 and co-incubated with diclofenac (1 µg/ml) showed no significant alterations of cytokine expression compared to the stimulated control, only a tendencially augmented TNF-α, IL-1β, IL-8, and GM-CSF expression was observed.

Shown are the mean values (± S.E.M.) after 18 h incubation at 36°C. *n*=3

The potently reduced PGE_2 secretion is likely to be linked to $cPLA_2$ and not to direct COX inhibition as the used concentrations for the ginger extract and 10-S were well under the published COX1/-2 IC_{50} values (*vide supra*). Additionally, the potency of PGE_2 inhibition is even higher than the inhibition of IL-1β processing and secretion in a similar assay setup. Whether this is an indication that eicosanoid synthesis plays a more important role for ginger on mediating anti-inflammatory activities *in vivo* is unclear.

The increased level of free intracellular AA upon ginger treatment could be explained as follows; free fatty acids (FFA) are first bound to CoA and then incorporated into lipids by either *de novo* synthesis of triglycerids (Kennedy pathway) or by remodelling of phospholipids (Lands cycle [395, 396]) (Fig. 22). The latter involves incorporation into phosphatidylcholine by a deacylation/reacylation reaction followed by a phospholipid class switch via CoA-independent transacylation [209, 385, 397] and it is the main pathway for arachidonic acid incorporation in most cell types [398]. In both of these processes

phospholipases, and especially $iPLA_2$, play major regulatory roles by providing FFA acceptor molecules (e.g. lyso-PC) [208]. Therefore, blockage of $iPLA_2$ deprives the cell of these acceptor molecules and would subsequently augment the intracellular FFA concentration [398]. On the other hand, immune cells generate free AA mainly from phospholipids by $cPLA_2$ IVA, the only PLA_2 with a preference for AA [233-236, 241], and only under certain conditions by $sPLA_2$ IIA, V, and X and $iPLA_2$ VIA [385]. The $iPLA_2$ inhibition is likely responsible for the increased free AA level whereas inhibition of $cPLA_2$ leads to decreased levels of eicosanoids.

The simultaneous potent inhibition of PGE_2 release and the significant increase in free AA in the macrophages (and the increased release into the culture medium) by ginger phenylpropanoids is in coherence with the finding that AA remodeling and eicosanoid synthesis are spatially separated processes.

That acetyl salicylate did only decrease PGE_2 without affecting free AA (Fig. 21) further supports the assumption that ginger constituents inhibit PGE_2 release not solely by COX inhibition. The combined inhibition of PLA_2 isoforms and eicosanoid biosynthesis, probably the combined LOX and COX inhibition previously reported [81, 82] or the interference with another molecular target (e.g. arachidonoyl-CoA synthetase [385]) (Fig. 22) seemed most likely. We therefore combined the $iPLA_2$ inhibitor BEL and the COX1/2 inhibitor acetyl salicylate which led to an increase of free AA similar to what was observed with ginger phenylpropanoids. We concluded that the reduction of lyso-PC, disrupting free AA incorporation, and the concurrent inhibition of COX isoenzymes is responsible for the unexpected effect on free AA by ginger phenylpropanoids.

The finding that MAFP at concentrations around the IC_{50} of reported i-/$cPLA_2$ inhibition [388, 399] only reduces free AA in the supernatant may be caused by the combined i-/$cPLA_2$ isoform inhibition and an indirect reduction of extracellular $sPLA_2$ activity. Moreover, one part of released free AA is rapidly reincorporated into phospholipids, whereas the other part is metabolised by β-oxidation or converted to eicosanoids [400, 401]. Unesterified AA in the plasma rapidly replenishes the intracellular pool, and this replacement is proportional to PLA_2 activation [402, 403]. As mentioned above, BEL alone inhibited free AA, but increased it when COX enzymes were inhibited at the same time. This change demonstrates the role of $iPLA_2$ in AA phospholipide reacylation. Consequently, concomitant inhibition of COX and $iPLA_2$ deprives the cell of phospholipid acceptor molecules and subsequently augments the intracellular free AA concentration [398].

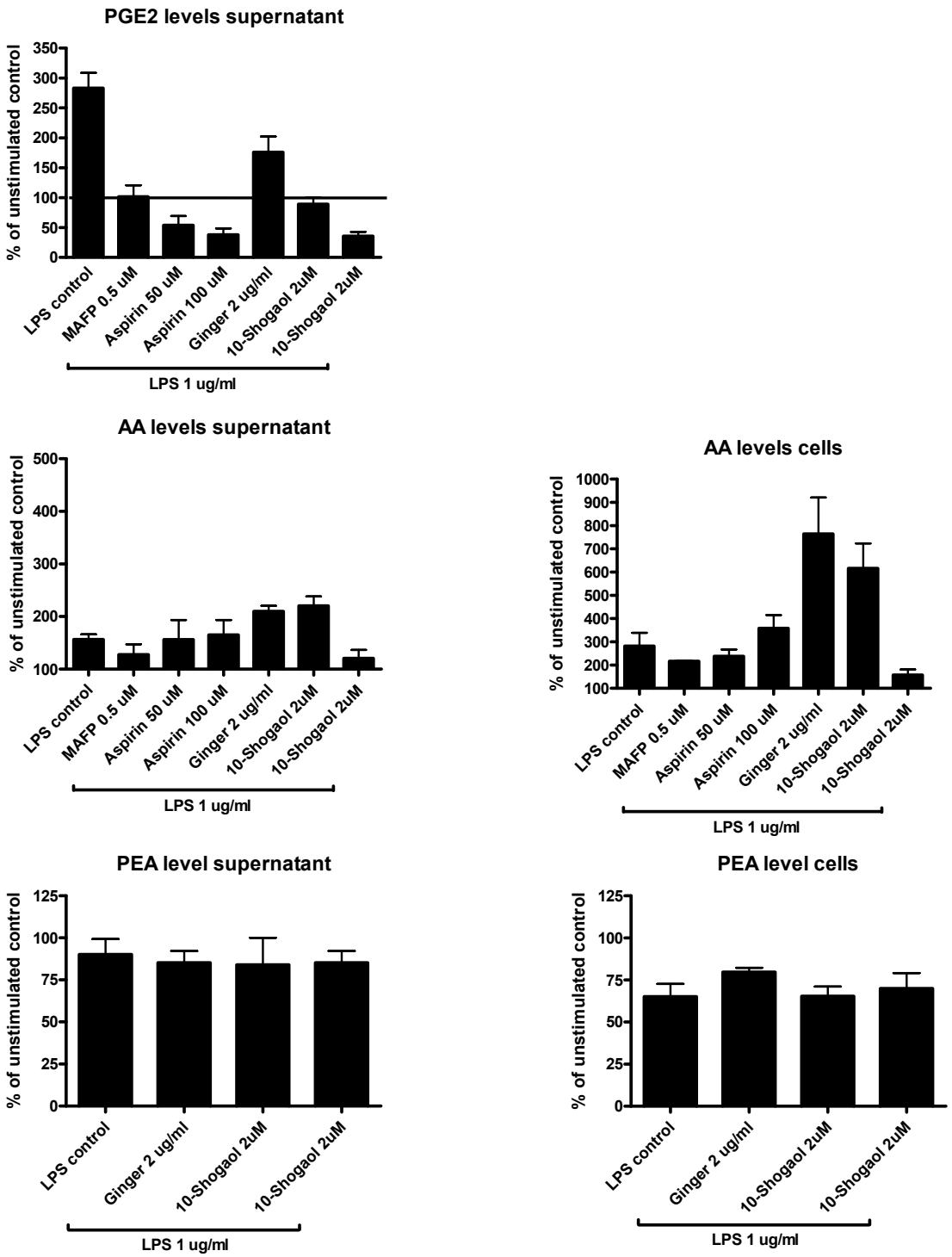


Figure 21: Effects of ginger extract and 10-shogaol on arachidonic acid, PEG_2, and PEA levels in LPS stimulated U-937 macrophages.

Supernatant levels of AA, PEG_2, and PEA and the intracellular level of AA was measured in PMA differentiated U-937 cells by GC-MC. Ginger Hot Flavor™ extract (Ginger, 2 µg/ml), 10-shogaol (2 µM), MAFP (0.5 µM) and acetyl salicylic acid (50 and 100 µM) were tested for their modulation of LPS stimulation. Indicated are the relative mean values + SD of triplicates compared to untreated controls; * $p<0.05$. Additional data have been published [328].

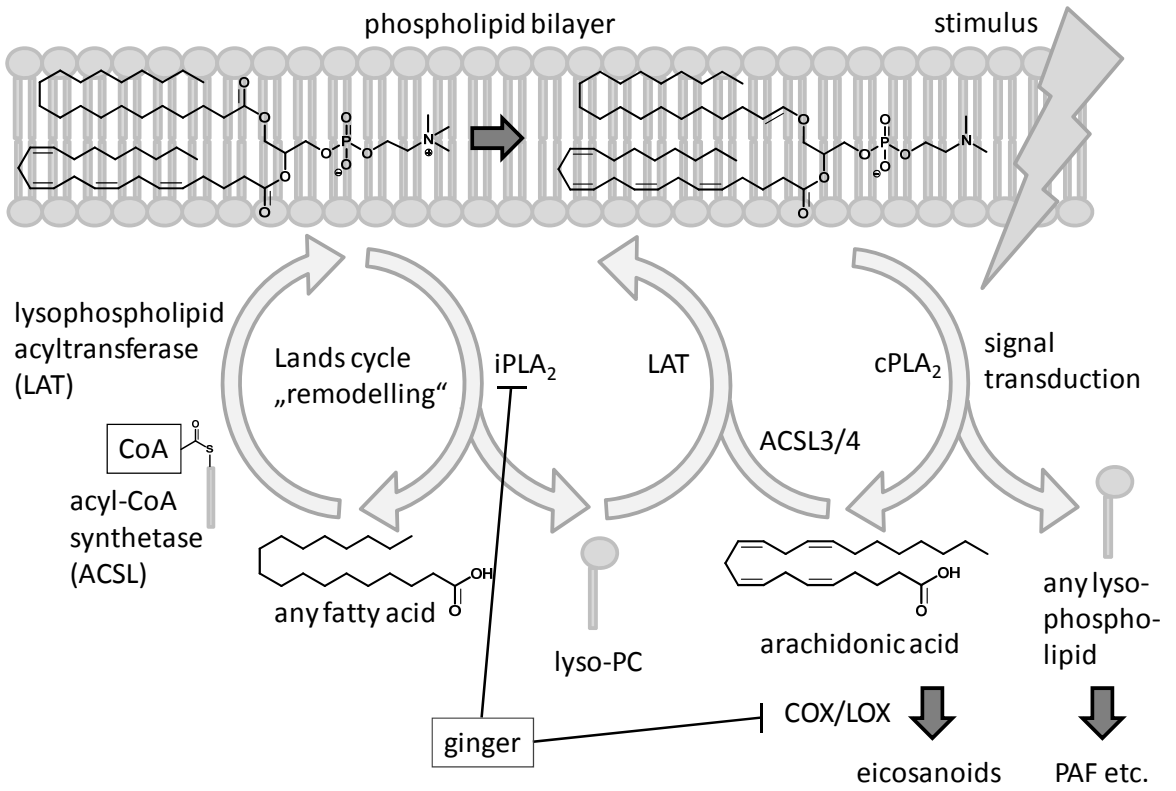


Figure 22: Phospholipid remodelling and PLA_2 signal transduction in immune cells.
Immune cells like monocytes/macrophages continuously re-organize arachidonic acid containing phospholipids in an $iPLA_2$-dependent manner (Lands cycle depicted on the left). Several stimuli activate $cPLA_2$-dependent signalling cascades (right side) [404]. Ginger phenylpropanoids, by the weak COX/LOX inhibition, the $_iPLA_2$ inhibition postulated in this work, and possibly other additional effects, seem to augment free arachidonic acid and simultaneously decrease PGE_2 generation thereby leading to a strong anti-inflammatory activity.

It is well known that free AA induces apoptosis in cells [220], which may also explain some of the observed antiproliferative effects of ginger and its constituents on cancer cells [405, 406]. Palmitoyl- and oleoylethanolamine (PEA and OEA, respectively) levels were not affected. Their biosynthesis mainly relies on N-acyltransferases (NAT), N-acylphosphatidylethanolamine phospholipase D (NAPE-PLD), and fatty acid acyl hydrolases (FAAH); Lyso-PLD and $sPLA_2$ are in volved to a minor extent, but no other PLA_2 classes are important [407, 408]. Consequently, our data affirm the selectivity of the ginger extract and its main constituents towards i-/$cPLA_2$ and exclude a non-specific toxicity, modulatory activities on endocannabinoid metabolism, and/or unspecific perturbation of lipid homeostasis.
The results from chapter 4.2.3 have been published [328].

4.2.4 Absorption Model

To gain some basic insight into the pharmacokinetic behaviour of ginger constituents we investigated the diffusion of the major gingerols and shogaols through a monolayer of Caco-2 cells, an in vitro cell culture system widely used to mimic the intestinal lining [409, 410] (Fig. 25). The ginger Hot Flavor™ extract was added at 50 µg/ml to either the apical or the basolateral side. Transport of the main constituents was analyzed in both configurations by an HPLC method developed to quantify gingerol-type constituents from cell culture medium at sufficiently high resolution (Fig. 23).

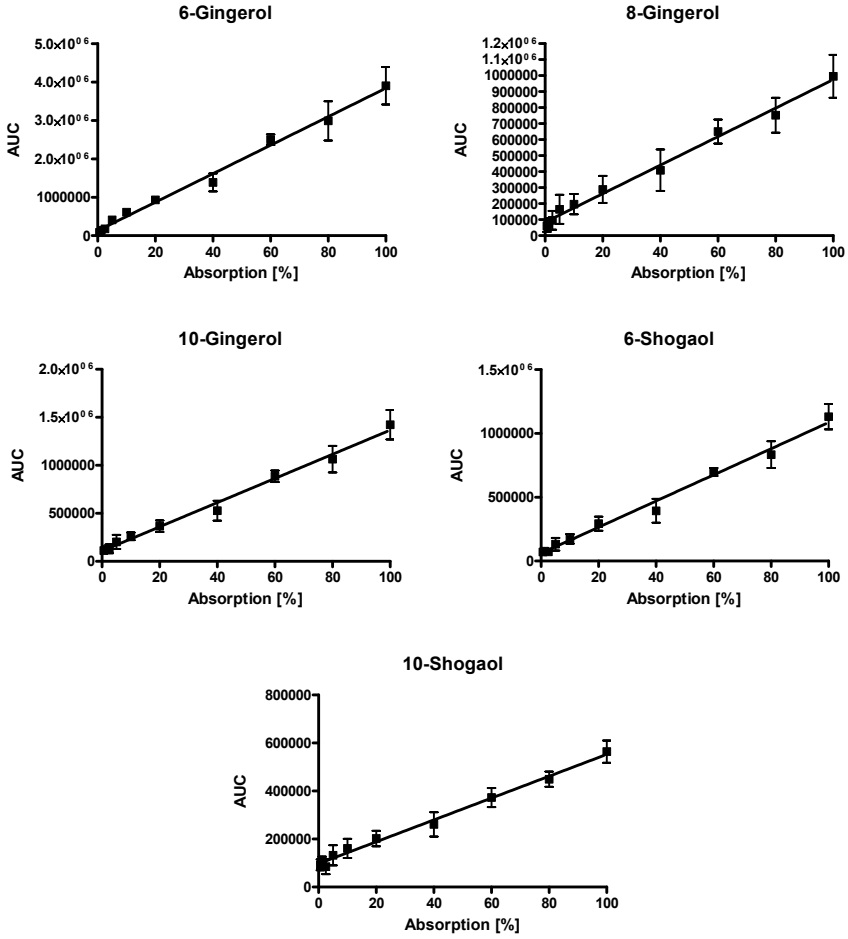


Figure 23: Calibration curves for the main constituents of ginger rhizome.

To determine the percentage of absorbed ginger phenylpropanoids, calibration curves from 0.625 to 100% (corresponding to 0.625 to 100 µg applied to the apical side of a Caco-2 assay well) for the main ginger constituents were generated on HPLC. Therefore, serial dilutions of the ginger Hot Flavor™ extract in HBSS were lyophilised, extracted with Et_2O, dried, dissolved in DMSO, and measured by HPLC with gradient C in strict analogy to the sample preparation. Given are the mean values of the area under the absorption curve (AUC) at 280 nm (squares) ± the 95% confidence interval of three independent serial dilutions and the linear regression (line).

The maximal non-cytotoxic concentration in this assay format was found to be 50 µg/ml (for 3 h). The integrity of the monolayer was monitored throughout the experiment by visual inspection and measuring trans-epithelial electrical resistance (TEER > 400 Ω/cm2). 6-, 8-, 10-gingerol and 10-shogaol crossed the Caco-2 monolayer by slow passive diffusion (30–60 % of the free compounds after 3 h, Fig. 24 A and B) while approximately 50 % was retained in the cellular monolayer. Therefore, the monolayer was extracted and analyzed too (Fig. 24 C).

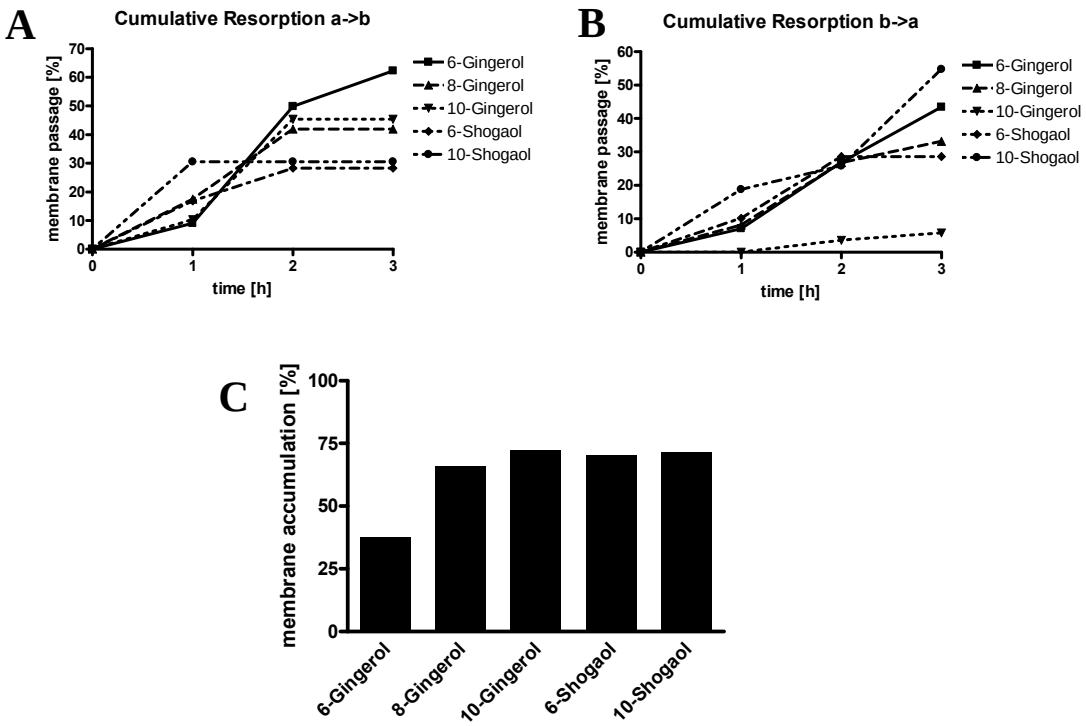


Figure 24: Caco-2 absorption assay.

Time-dependent passage of major ginger constituents (50 µg/ml) across Caco-2 monolayers (A) from apical to basolateral and (B) from basolateral to apical. Used formula: 100 * amount basolateral / (amount apical + basolateral).

C Accumulation of all compounds, except 6-gingerol, in the Caco-2 cells was high (~ 70 %) and equal for both directions.

Shown are the mean values of three independent experiments each done in a triplicate. SD was less than 15 % of the corresponding value.

6-gingerol diffused across the monolayer with significantly higher efficiency than its dehydrated counterpart 6-shogaol (Fig. 24 A and B), in spite of it higher polarity. However, the incubation time in this assay was limited by both membrane effects and the sensitivity of the HPLC method.

The apparent permeability of the determined compounds is likely to be underestimated in the used Caco-2 assay due to retention by the cell-layer. To circumvent concentration dependant cell accumulation, an absorption assay using pure compounds at equal concentrations would be favourable.

8-Shogaol could not be quantified due to the overlap with a peak of a buffer constituent. This might be corrected by assay modifications using for example up to 10 % DMSO in the apical compartment with the necessity of BSA or similar on the basolateral side [411, 412]. Alternatively, a LC-MS method could be employed.

4.2.5 Caco-2 Whole Blood Co-culture Assay

The suitability of the Caco-2 co-culture with diluted human blood in the basolateral compartment (Fig. 25) as tool for assaying anti-inflammatory properties of ginger preparations was examined.

In addition to tight monolayers with a TEER exceeding 400 Ω/cm^2 as used for gingerol absorption studies monolayers with a low TEER of around 200 Ω/cm^2 ('leaky' monolayer) were used as controls.

In accordance with the findings in whole blood, stimulation with phorbol ester plus the CD3 antibody of HBSS diluted blood alone or in the presence of a Caco-2 monolayer separated by a semi-permeable membrane resulted in a strong induction of the measured cytokines (e.g. TNF-α, IL-10, IL-1β, IL-6, and IL-8) (Fig. 26 A). The expression in the co-culture was lower, either due to dilution by the introduction of an additional apical compartment or due to an inhibition caused by the Caco-2 cells as proposed in some reports [413-416]. Ginger Hot Flavor™ extract in stimulated diluted blood at 50 μg/ml (in analogy to the whole blood assay) and at 10 μg/ml (the theoretical equilibrium concentration in the co-culture) resulted in a dose-dependent inhibition of cytokine expression (Fig. 26 B and C). Unexpected were the results from the co-culture in which the extract was added (at 50 μg/ml) to the apical side with stimulated diluted blood on the basolateral one. Expression levels of all aforementioned cytokines were slightly elevated (Fig. 26 B and C; $p \leq 0.05$ for TNF-α and IL-10) with tight monolayers.

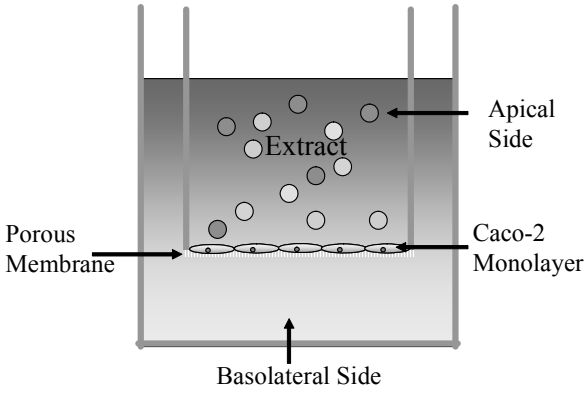


Figure 25: Absorption Model.

A collagen coated insert with a porous membrane (pore size 0.4 µm) was placed into a bigger well and a Caco-2 monolayer was grown in it. The resulting tight and polarized monolayer mimics the intestinal lining wherein the apical compartment corresponds to the luminal side of the gastrointestinal tract. Absorption is usually estimated by applying the compounds of interest to the apical side followed by the determination of their concentration over time on the basolateral side. Instead, we exchanged the basolateral buffer with whole blood and determining cytokine expression after 18 h incubation at 37 °C in both compartments.

The desired co-culture of Caco-2 cells with fresh whole blood could be established but it was not suitable for the determination of anti-inflammatory activities of ginger extracts. This could be explained by the ability of Caco-2 cells to inhibit the cytokine expression by blood cells.

The mechanism by which the intestinal epithelial cells inhibit monocyte and T cell function is unclear and might be mediated by prostaglandins [415, 417, 418]. Because ginger constituents do not reach high enough concentrations in the basolateral compartment (see chapter 4.2.2) they might only have an inhibitory effect on the Caco-2 cells resulting finally in an increased cytokine expression by whole blood cells. Maybe the diffusion kinetics is too slow and not enough of the compounds are able to reach the site of action. Thus, a prolonged pre-incubation time may have different effects.

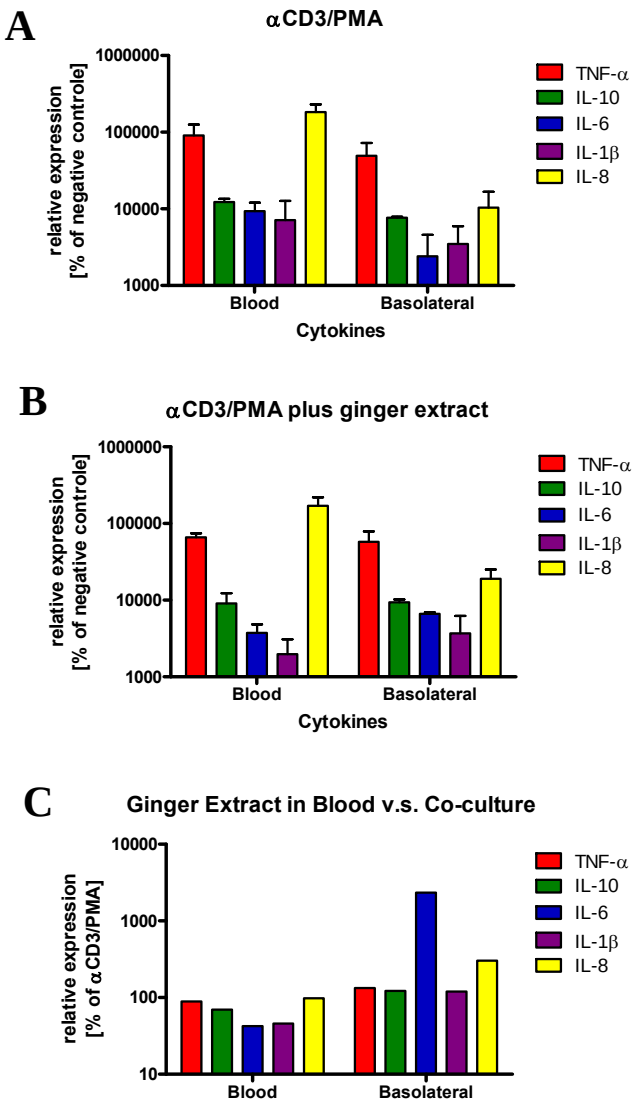


Figure 26: Cytokine expression in the basolateral compartment of the Caco-2 co-culture and in whole blood.

A Diluted human blood was stimulated with PMA and αCD3 for 18 hours either alone (left) or in the co-culture (right) resulting in a strong cytokine induction. In the co-culture the expression was 1.5 to 3 fold lower for all cytokines except IL-8. **B** Ginger Hot Flavor™ extract added to the blood at 10 µg/ml dose dependently reduced the expression of all cytokines. In contrast, when added to the apical side at 50 µg/ml, cytokine expression was augmented by 1.1 to 3 fold. **C** Relative expression pattern of the ginger Hot Flavor™ extract in whole blood and in the co-culture. In the former cytokine expression was inhibited whereas in the latter it was enhanced.

The results are the mean of three independent measurements done in duplicates plus the 95 % confidence interval. Y-values are given in % of the corresponding vehicle controls.

4.2.6 Interaction of Gingerols and Shogaols with P-glycoprotein

Since P-glycoprotein (Pgp) substrates generally show a limited passage across the blood brain barrier we assessed whether gingerols and shogaols were able to competitively inhibit the efflux of rh123 in human Pgp transfected MDCK cells. Neither gingerols nor shogaols either alone or in combination were found to act as substrates for Pgp up to a concentration up to 30 μM (Fig. 27). Unlike the positive controls verapamil (which is used as a Pgp inhibitor) or vinblastine (a classical Pgp substrate) the ginger compounds did not inhibit rh123 efflux. The slightly increased efflux observed (Fig. 27) is likely due to non-specific membrane effects of the lipophilic gingerols and shogaols. [332]

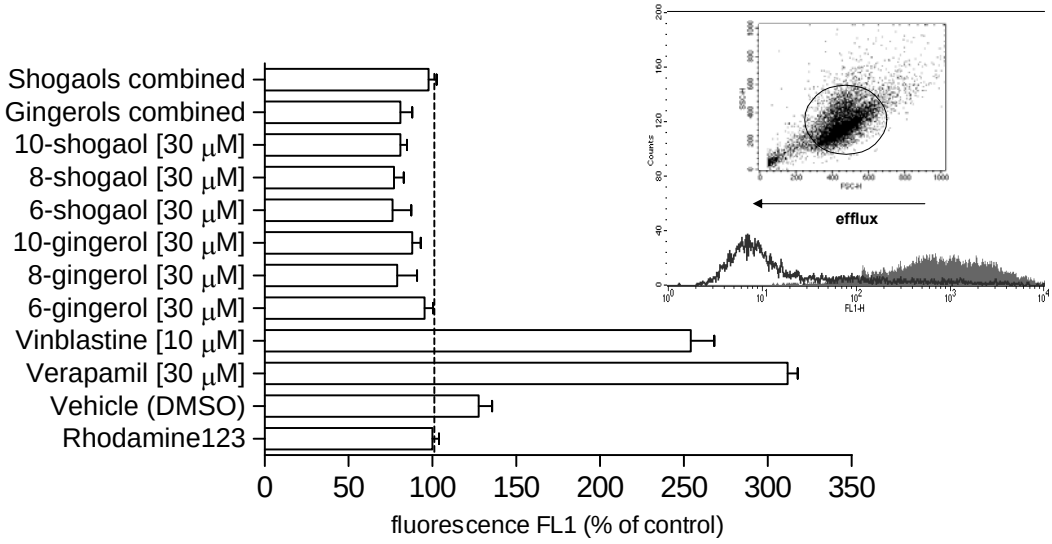


Figure 27: Effects of shogaols and gingerols on Pgp-mediated rhodamine123 (10 μM) efflux from MDCK cells ($2 \cdot 10^6$ cells) stably transfected with human Pgp.

Shown is the mean fluorescence measured (geometric mean in FACS, 10^4 cells counted, see histogram insert) as % of remaining cellular rhodamine123 after washing, relative to control. The Pgp inhibitor verapamil and the Pgp substrate vinblastine were used as positive controls.

Data show mean values of 3 independent experiments ± SD

4.2.7 Conclusions

We started our investigations with human whole blood as a complex model simulating physiological conditions. Fresh human blood was stimulated with a set of stimuli targeted against different subsets of leukocytes and as readout eleven cytokines were quantified resulting in different expression patterns. Using whole blood from different donors showed that the assay could be used to detect the robust changes in cytokine expression induced by discrete stimuli and inhibitors. Thus, the assay was less susceptible to artefactual modulations often observed in cellular assays. The most noticeable finding was that ginger

extracts rather specifically inhibited IL-1β expression, nearly independent of the used stimuli. This led to the hypothesis that certain ginger constituents were able to interfere with either a signalling pathway exclusively leading to IL-1β gene expression or more likely a target downstream of this.

Detection of pro-IL-1β and mature IL-1β by electrophoresis and Western blot was performed by means of a well established assay with LPS primed human monocytes. A significant inhibition of cytokine transcription/translation could not be detected but an inhibition of processing and secretion. These latter two processes are regulated by ion fluxes, caspase-1, and two forms of phospholipases A_2. We could show that ion fluxes were not affected and that both PLA_2s were functionally inhibited. In addition, Shin et al. [419] isolated a diarylheptanoid from *Alpinia officinarum*, structurally related to gingerol-type constituents, which inhibits pancreatic lipase. Therefore we focused our attention on PLA_2 and not on caspase-1.

Our data show that ginger extracts and the main constituents of the oleoresin exert an anti-inflammatory activity in human whole blood and isolated monocytes at least in part by inhibiting i- and $cPLA_2$. Inhibition of these enzymes interferes with the Lands cycle [395, 396] and leads to deprivation of free fatty acids as a substrate for COX and LOX enzymes within the cell. Our findings are in line with the already known antioxidant and radical scavenging activities of ginger [102, 104, 420] which may further increase the effects on $iPLA_2$ in cells; reactive oxygen species produced by cyclo-/lipoxygenases are known to either activate $iPLA_2$ in a positive feed-back loop [339] or act as mediator between $cPLA_2$ and $sPLA_2$ [243, 421, 422]. An indirect inhibition of $sPLA_2$ *in vivo* might also result from $cPLA_2$ inhibition [422].

Inhibition of PLA_2 could be the rational basis for several indications for ginger (e.g. inflammatory diseases like arthritis, illnesses of the airways, fever, pain, or cardiovascular disorders [405, 406]) and experimental effects (e.g. inhibition of PGE2 and cytokine production in isolated cells, rodent paw edema formation, or skin cancer progression). Even the effect of ginger preparations on blood coagulation and cancer cell progression, both PLA_2-dependent mechanisms, could be explained.

Ginger rhizome and its extracts have been shown to be safe in use and reported weak adverse effects are typically associated with its pungency. In contrast, many tested synthetic PLA_2 inhibitors exert unwanted side-effects due to unspecific toxicity/reactivity (e.g. BEL or MAFP), poor selectivity (e.g. MAFP and arachidonyl trifluoromethyl ketone), lacking

oral availability (e.g. EXPLIS [244]) or non-sufficient activity *in vivo* [272]. Ginger extracts or isolated constituents show similar *in vitro* potencies against PLA_2 as standard PLA_2-inhibitors but exert additional beneficial effects like antioxidant and radical scavenging properties, which might enhance/support ginger's anti-inflammatory activity [243, 339, 420-423]. Furthermore ginger as a phytomedicine has great acceptance in the population and might therefore be used as a physically and mentally well-tolerated augmentation therapy to conventional anti-inflammatory medication. High protein binding, fast blood elimination (in mice [54-56]), suboptimal physical properties (e.g. solubility), but promising animal studies (sunburn [90] and paw and skin edema [41]) also suggest the use as topical application on the skin (ginger plaster [424]), in the ear (against inner ear disease [425]), or the intestinal tract mucosa (in particular for the treatment of inflammatory bowel disease [426] and celiac disease [427]) even if not used traditionally as such.

4.3 Assessment of Serotonergic Effects of Ginger Extracts

4.3.1 Bioactivity-guided Isolation of 5-HT_{1A} Receptor Ligands

For an appropriate bioactivity-guided isolation, several TLC methods were evaluated to determine optimal separation conditions.

TLC with n-hexane/diethyl ether/ethyl acetate (2:2:1) gave the following R_f-values: proteins/sugars 0-0.3; gingerols 0.3-0.38 (254 nm); shogaols 0.6 (254 nm); yellow fluorescence band 0.65 (366 nm); probably diarylheptanoids at 0.75-0.85 (366 nm); terpenes at 0.75-1 (254 nm). TLC with n-hexane/diethyl ether/acetic acid (20:20:1) gave similar R_f-values but a better spot separation.

TLC with n-hexane/diethyl ether/ethyl acetate/acetic acid (40:40:20:0.5) gave a better resolution and slightly higher R_f-values: proteins/sugars 0-0.2; 6-gingerol 0.4 (254 nm); 10-gingerol 0.45 (254 nm); 6-shogaol 0.65 (254 nm); yellow fluorescence band 0.7-0.75 (366 nm); probably diarylheptanoids at 0.8-0.9 (366 nm); terpenes at 0.8-1. Gingerols and shogaols were determined with standards and with reduction selective development methods (e.g. Tollens and chloranil reagents).

The presence of sugars and proteins in alcoholic extracts and their absence in the CO_2 extract was demonstrated with ninhydrin and diphenylamine/aniline detection.

Based on the hypothesis that certain ginger constituents may be able to modulate the 5-HT_{1A}R, the commercial food grade ginger Hot Flavor™ extract was tested for displacement of the high-affinity 5-HT_{1A}R -selective ligand [^{3}H]-DPAT and the 5-HT_3R -selective ligand [^{3}H]-GR65630 from membranes obtained from HEK-293 cells transfected with serotonin 5-HT_{1A} and 5-HT_3 receptors, respectively. In agreement with a previous report [35], the ginger extract tested did not show a significant displacement (>50 % at 25 μg/ml) of the radioligand from the 5-HT_3 cation channel (data not shown). In contrast, the extract and major fractions thereof strongly displaced [^{3}H]-DPAT from the 5-HT_{1A}R (Fig. 28 A), with a K_i value for the whole extract of 11.57 ± 1.56 μg/ml (Fig. 28 B and C).

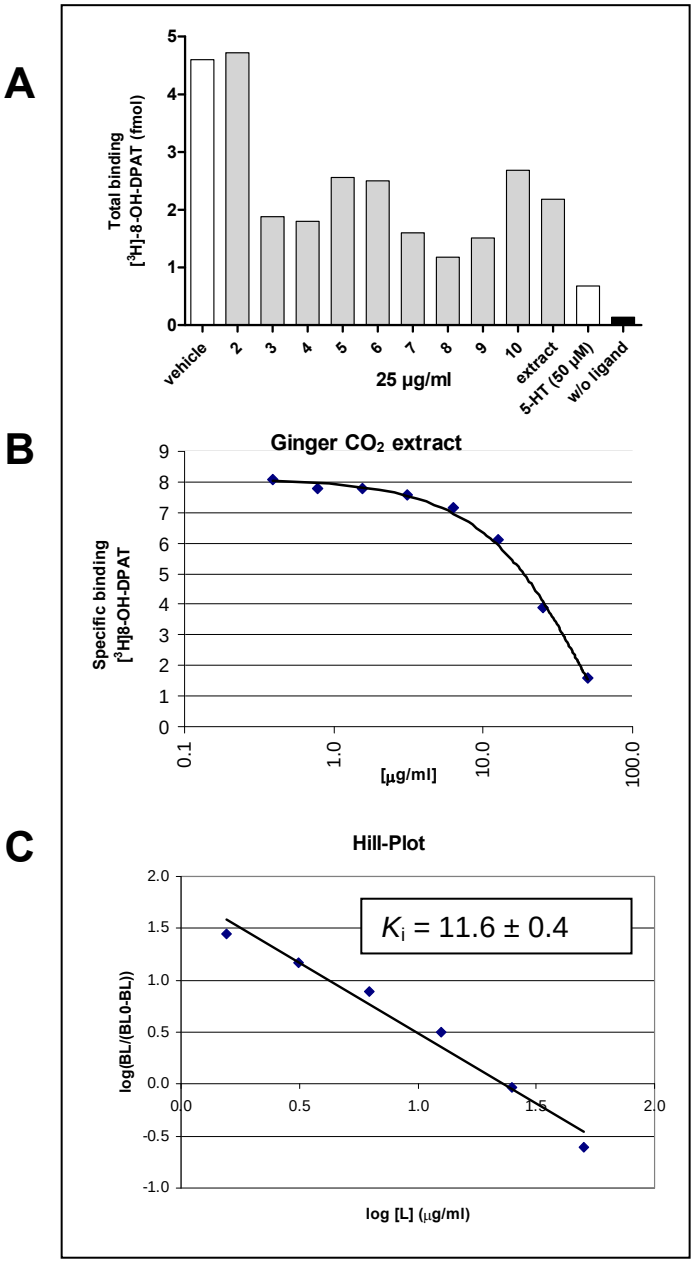


Figure 28: Bioactivity guided fractionation of 5-HT$_{1A}$R ligands from ginger rhizome.

A Displacement of [^{3}H]-DPAT by the ginger Hot Flavor™ extract (extract) and chromatographic fractions (2–10). Serotonin (5-HT) was used as a positive control (50 µM), **B** concentration-dependent displacement of [^{3}H]-DPAT by ginger Hot Flavor™ extract, **C** linearised data in Hill plot and corresponding inhibition constant (K_i value).

Data show mean values of 3 independent experiments.

Ginger extract at 25 µg/ml also displaced the high-affinity ligand [^{3}H]-RTX (resiniferatoxin) from the transient receptor potential V1 (TRPV1) channel (approximately 50 % displacement), a calcium channel already known to be a target for the spicy gingerols and shogaols [93] (Fig. 29). In contrast, it did not significantly displace [^{3}H]-CP55,940 from the cannabinoid type-2 receptor (CB2) or [^{3}H]-fMLP from the formyl peptide like-1 receptor (FPRL1) (Fig. 29), both of which are GPCRs.

Ginger essential oil did not displace the radioligand (not shown), thus excluding most mono- and sesquiterpenes as ligands. Furthermore no non-specific cell membrane effects of these lipophilic compounds could be seen. Although limited to a few selected cases, these binding data indicated that the ginger extract did not bind indiscriminately to just any membrane-embedded receptor and this finding provided the impetus for a more detailed investigation of the interactions of individual ginger constituents with the 5-HT$_{1A}$R.

For this purpose we undertook an iterative bioactivity-guided fractionation of the ginger Hot Flavor™ extract employing flash liquid chromatography (flash LC) and high-performance liquid chromatography (HPLC) (Fig. 30), which finally led to the isolation of 9 compounds exhibiting significant displacement of [^{3}H]-DPAT from the 5-HT$_{1A}$R (>50 % displacement at 25 µg/ml).

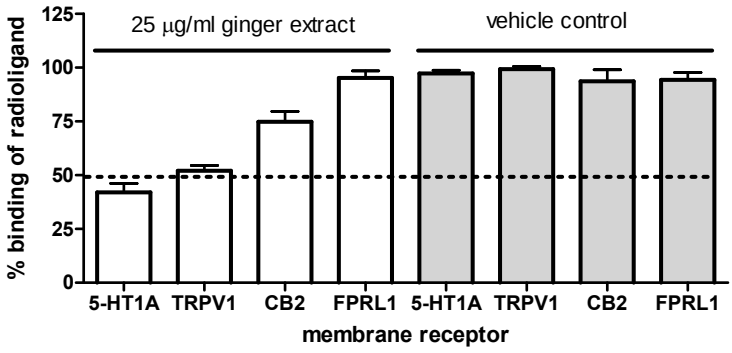


Figure 29: Displacement of selective radioligands from different membrane proteins.

Ginger Hot Flavor™ extract (25 µg/ml) was tested for displacement of corresponding radioligands from several membrane receptors (5-HT$_{1A}$ receptor; 5-HT1A, transient receptor potential V1; TRPV1, cannabinoid receptor type 2; CB2, formyl peptide like-1 receptor; FPRL-1). Data are mean values of 3 independent experiments ± SD

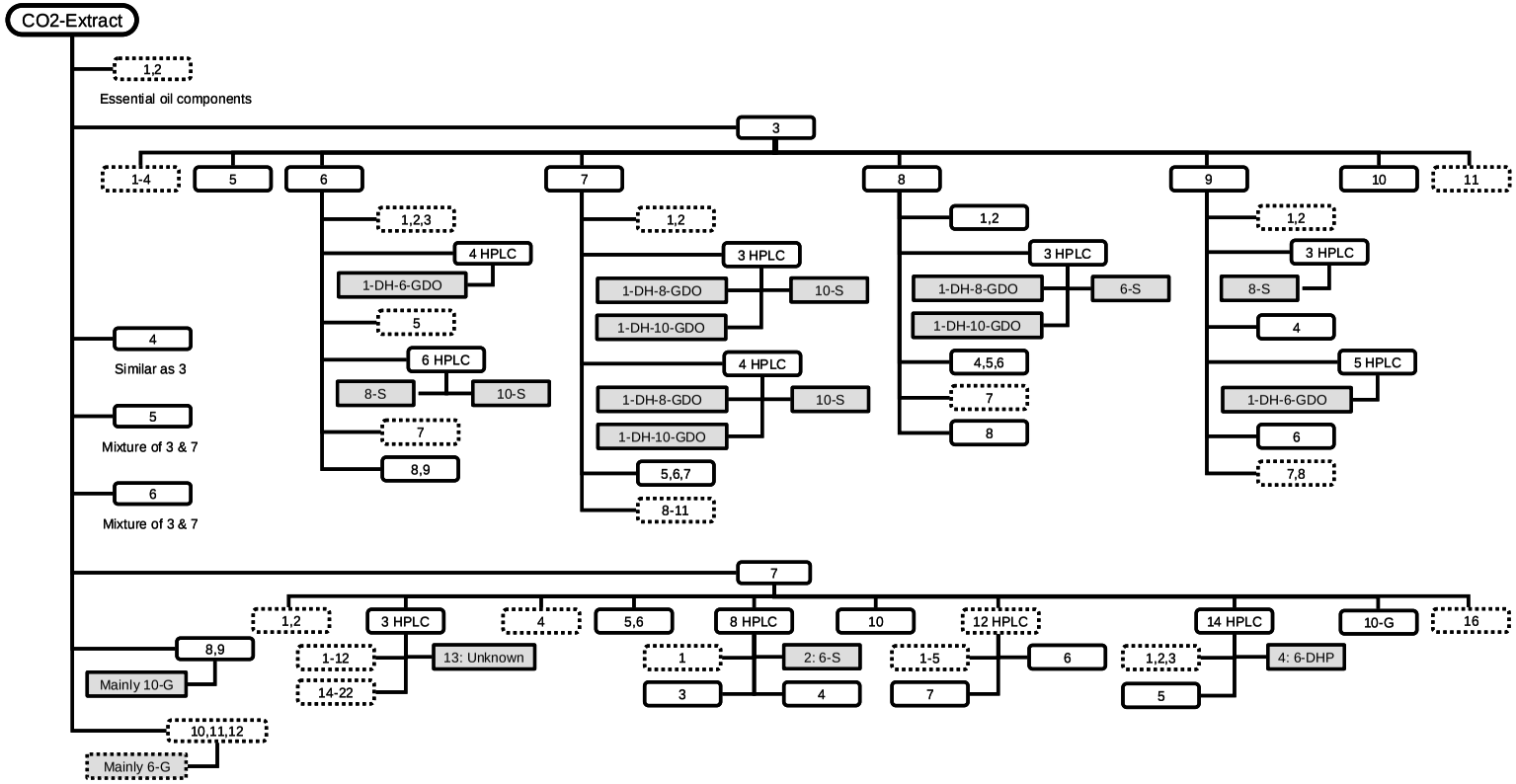


Figure 30: Bioactivity-guided isolation procedure.

Ginger Hot Flavor™ extract was subjected to flash column chromatography and HPLC and fractions showing >50% displacement of [^{3}H]-DPAT in the screening (dark lines) were further purified to yield pure compounds (grey squared boxes). Inactive fractions or compounds are

Chemical structure	Extracts and isolated compounds	K_i ± SD (µM)	K_i ± SD (µg/ml)
	Ginger Hot Flavor™ extract		11.57 ± 1.56
	Ginger essential oil		>50
	6-gingerol (n=1)	>100	>50
	8-gingerol (n=2)	11.71 ± 4.35	3.77
	10-gingerol (n=3)	3.74 ± 1.77	1.31
	6-shogaol (n=1)	18.02 ± 4.77	4.97
	8-shogaol (n=2)	13.05 ± 1.19	3.97
	10-shogaol (n=3)	5.84 ± 1.14	1.94
	1-dehydro-6-gingerdione (n=1)	6.53 ± 0.72	1.89
	1-dehydro-8-gingerdione (n=2)	6.54 ± 1.35	2.08
	1-dehydro-10-gingerdione (n=3)	3.59 ± 1.08	1.24
	6-dihydroparadol	9.53 ± 0.70	2.67

Table 6: Binding affinities to the 5-HT$_{1A}$R

Inhibition constant (K_i) values were determined by displacement of [^{3}H]-DPAT from receptor- transfected HEK-293 cells. Data were obtained in at least 3 independent experiments. In this assay the K_i value for serotonin was <50 nM

These compounds (Table 6) were characterized by spectroscopic and spectrometric means, including 2D and 3D nuclear magnetic resonance (NMR) measurements and mass spectrometry (ESI-MS). They were also compared to reference spectra [14, 18, 428-436] and commercial samples, if available. Based on the K_i values obtained for all major compounds in the radioactive ligand displacement assay (Table 6), 6-gingerol does not bind to the 5-HT_{1A}R with any measurable affinity, while K_i values between 3 and 20 μM were determined for all other compounds. Overall, binding appears to correlate with the length of the aliphatic tail, with longer chains leading to higher affinity and 10-gingerol, 10-shogaol and 1-dehydro-10-gingerdione (1-DH-6-GDO) being the strongest binders (Table 6). These results have been published [332].

Most serotonin receptor ligands of biological origin are indoles or phenylethylamines, and thus contain at least one nitrogen atom, which is important for binding affinity. Besides valerenic acid [437], asparvenone-derivatives [438], or butenolides from *Piper hispidum* [439], gingerol derivatives are among the rare nitrogen-free serotonin receptor ligands known to date.

4.3.2 5-HT_{1A} Receptor Activity

In order to address the question whether the ginger extract and its 5-HT_{1A}R binding constituents are able to functionally modulate the 5-HT_{1A}R, 1-50 μg/ml of the ginger extract and 10 μM of each compound were analyzed in [^{35}S]-GTPγS assays, both alone and in combination with DPAT and serotonin. As expected, in this assay 10 μM of serotonin, which yielded a K_i value <50 nM in the displacement assay, induced approximately 100 % of the maximal effect on [^{35}S]-GTPγS binding that is achievable with DPAT (Fig. 31 left).

The ginger extract potently increased [^{35}S]-GTPγS binding (approximately 40 % of the maximum effect of DPAT) at concentrations as low as 10 μg/ml, while 50 μg/ml led to membrane-toxic effects and the disruption of [^{35}S]-GTPγS binding (Fig. 31 left). For the individual ginger constituents effects >10 % relative to the maximal effect induced by DPAT (100 %) were only observed with 10-gingerol, 6-dihydroparadol, 10-shogaol, and 1-DH-6-GDO (Fig. 31 right). The two latter compounds showed partial agonism (>20 %) and where therefore subjected to a concentration-effect analysis (Fig. 32 A and B).

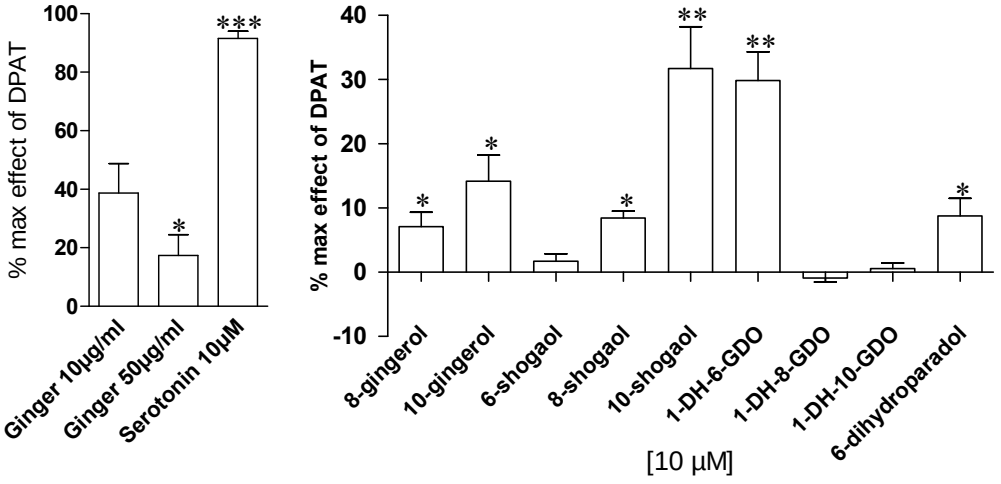


Figure 31: Effect of ginger extracts and ginger-derived isolated $5HT_{1A}R$ ligands on G_i activation in 5-$HT_{1A}R$ transfected HeLa cells as assessed by [^{35}S]-GTPγS binding.

Activation efficiency is relative to the maximum effect achievable with DPAT; DH-DGO = 1-dehydro-6-gingerdione. Data are mean values of at least three independent experiments using three membrane preparations (±S.D). * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.005$.

Unfortunately, these experiments were hampered by the fact that 10-shogaol and 1-DH-6-GDO apparently altered membrane integrity in a non-specific way, thus leading to the disappearance of [^{35}S]-GTPγS binding at concentrations above 20 µM (Fig. 32 A and B). Consequently, the maximum effects remain unknown for 10-shogaol and 1-DH-6-GDO and it was not possible in this assay format to differentiate between partial and full agonism. 8-, and 10-gingerol, 8-shogaol, and 6-dihydroparadol all behaved as weak partial agonists, while 6-shogaol, 1-DH-8-GDO, and 1-DH-10-GDO did not show any intrinsic activity.

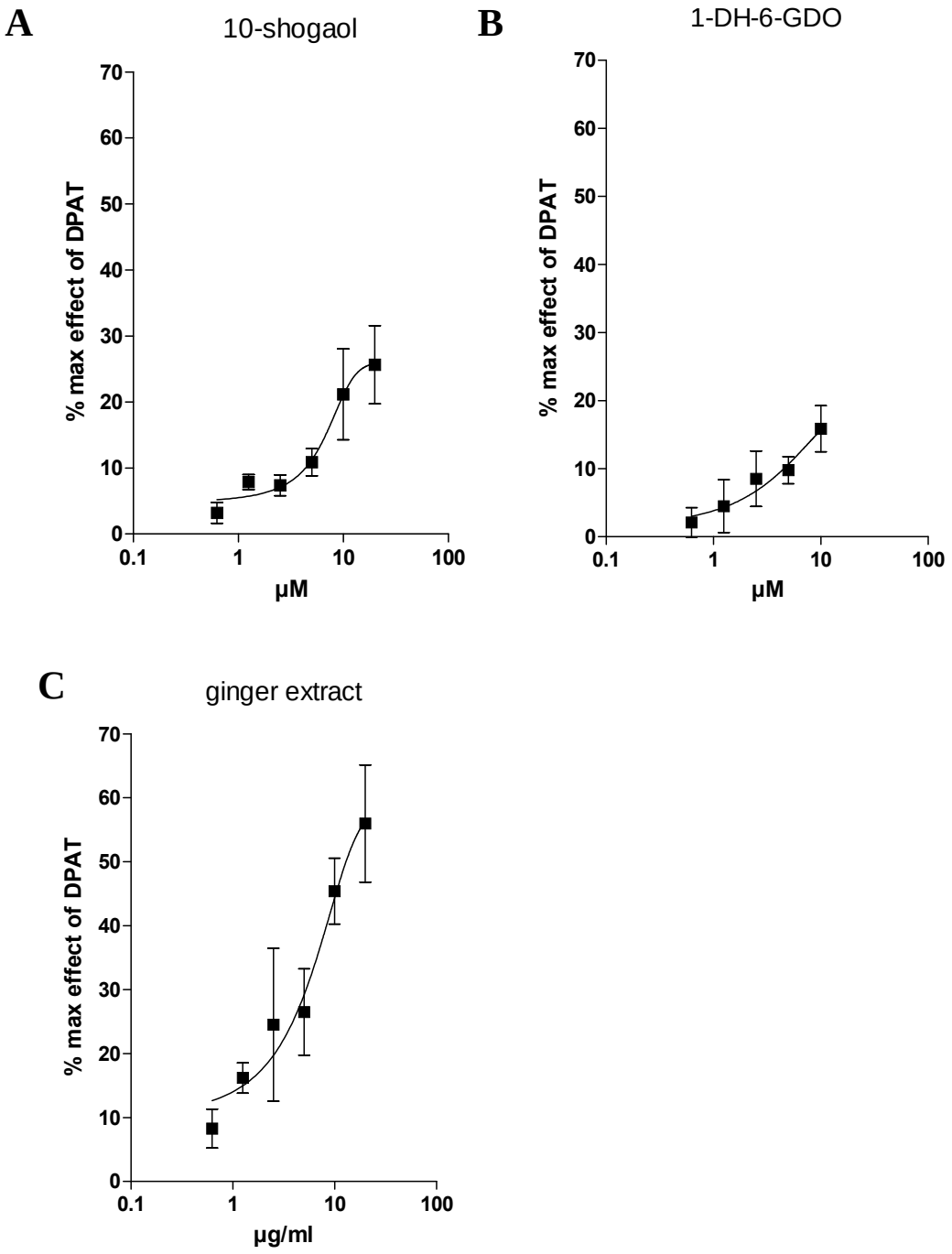


Figure 32: Functional effects in 5-HT_{1A}R transfected HeLa cells.

Concentration-dependent action on [^{35}S]-GTPγS binding: **A** 10-shogaol, **B** 1-dehydro-6-gingerdione (1-DH-6-GDO). At concentrations above 20 µM the assay became dysfunctional. Data obtained with concentrations >20 µM could therefore be misleading and the maximum effects of the compounds cannot not be determined. **C** The most potent induction of [^{35}S]-GTPγS binding was observed for the ginger Hot Flavor™ extract, which led to 60 % activation at the highest concentration tested. Data are mean values of 3 independent experiments ± SD

None of the compounds tested was able to significantly modulate [^{35}S]-GTPγS binding induced by DPAT or serotonin (data not shown). Intriguingly, the whole ginger extract not only induced a concentration-dependent activation of the 5-HT_{1A}R, but the magnitude of the effect was significantly more pronounced than for any of its individual constituents (at the same w/v concentration), reaching as much as 60 % [^{35}S]-GTPγS binding (relative to the maximum DPAT effect) at a concentration as low as 20 μg/ml (Fig. 32 C). This clearly suggests that the different components of the whole ginger extract may act in an additive or even super-additive manner at a functional level.

Several complementary and self-helped treatments are used against anxiety disorders with ginger being one of them (as combination in *kampo* herbal preparations) even though its effectiveness is weak and may not be attributed to ginger itself [440].

4.3.3 Serotonin in Whole Blood

Potential effects of ginger constituents and of the 5-HT_{1A}R in inflammatory reactions were elucidated in the blood assay. First, the selective 5-HT_{1A}R agonist DPAT and the selective 5-HT_{1A}R antagonist NAN-190 were tested in PMA/αCD3 stimulated whole blood, each at 100 nM and 100 μM. Their cytokine profiles were compared with the one of ginger Hot Flavor™ extract (at 50 μg/ml) (Fig. 33). Only 100 μM resulted in a statistically significant modulation of cytokine expression (Fig. 31) which might be due to high endogenous serotonin concentration. The modulation pattern observed for the ginger extract does not match either of the patterns obtained with the selective 5-HT_{1A}R antagonist nor the same one as the agonist.

The antagonist NAN-190 has a pro-inflammatory activity by enhancing the expression of TNF-α, IL-6, and IL-1β and concomitantly inhibiting IL-10, whereas the agonist marginally induces TNF-α but mainly inhibits IL-1β expression; ginger Hot Flavor™ extract inhibits TNF-α, IL-6, IL-1β, and IL-10. 5-HT_{1A}R ligands exert somewhat contradictory results in the whole blood assay compared with literature data. 5-HT_{1A}R antagonists and other cAMP elevating agents are linked to an inhibition of lymphocyte function, T_H1 cytokine expression (mainly IL-2 via inhibition of NFAT), and proximal TCR signal transduction [441, 442].

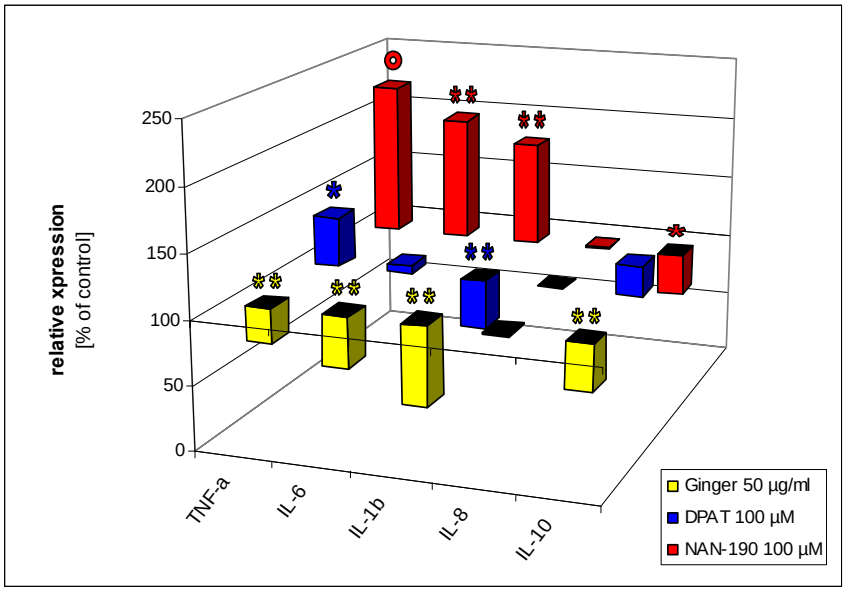


Figure 33: Modulation of cytokine expression in PMA/αCD3 stimulated human whole blood by 5-HT$_{1A}$ receptor ligands.

Shown are the relative expression patterns of inflammatory related cytokines when treated with either ginger Hot Flavor™ extract (ginger), the 5-HT$_{1A}$R agonist (DPAT) or antagonist (NAN-190) compared with only stimulated blood after 18 h incubation.

The data are the mean values of at least four independent experiments with blood of different donors. * $P \leq 0.10$; ** $P \leq 0.05$; ° $P \leq 0.01$ when not counting the outlier which has the highest expression level.

This is caused by the inhibition of the $\alpha_{i/0}$ and β/γ coupled signal transduction resulting in the former case in a transiently increased level of cytosolic cAMP and subsequent activation of protein kinase A (PKA) and, in the latter case, in reduced PLCβ/γ activity. On the other hand, PKA is able to enhance the nuclear translocation of p38, ERK1/2, and NF-κB, three pathways linked to pro-inflammatory cytokine expression. In accordance with the activation of these pathways our own findings show that the cAMP elevating compounds NAN-190 and forskolin (data not shown) lead to an increased expression of TNF-α, IL-6, IL-1β, and IL-8 in PMA/αCD3 stimulated whole blood. The activation of the G_i coupled fMLP receptor caused similar effects too but is likely to be caused by the oxidative burst of neutrophils [443]. In contrast, the agonist DPAT caused a significant decrease in IL-1β expression only at a concentration at which selectivity is lost and binding also occurs to other 5-HTR subtypes like the G_s coupled 5-HT$_7$R.

These results show the difficulty of examining serotonergic effects in whole blood with a constitutive serotonin concentration of 20 to 100 nM which, under inflammatory conditions or pharmacologic intervention, may reach 100 to over 1'000 nM [184]. This might explain the required high concentration of the antagonist NAN-190 and the ineffectiveness of the selective agonist DPAT to modulate cytokine expression in stimulated whole blood.
Furthermore, the data demonstrate that the interpretation of results gained from whole blood assays have to be considered with care and are off the limitations especially when the blood is additionally treated with a 'broad-band' stimulus like the PMA/αCD3 combination. As for of ginger extracts no conclusions could be drawn regarding serotonergic effects in stimulated whole blood, but it seems likely that the anti-inflammatory effect is caused by another effect than the partial agonism at the 5-HT_{1A}R.

4.3.4 Conclusions

Ginger rhizome contains several phenylpropanoids which bind to the 5-HT_{1A}R and act as weak partial agonists as determined by [^{35}S]-GTPγS binding. Interestingly, the IC_{50} value for the extract and its constituents, determined by radiolabelled ligand displacement, suggests an additive effect of the individual compounds in the extract. In contrast, the activity of the extract at 20 µg/ml reaches 60% of the maximal effect, which cannot be explained by the activity of its constituents, which barely reached 30% activity (e.g. 10-gingerol, 10-shogaol, and 1-dehydro-6-gingerdione). This finding is likely caused by the physical properties of the pure compounds, which interfered with the [^{35}S]-GTPγS assay at 10 to 20 µM. Noteworthy is the finding, that the main constituent 6-gingerol shows no affinity for the 5-HT_{1A}R. Pharmacological effects with regard to inflammation mediated by this receptor were not found; a selective 5-HT_{1A}R agonist and an antagonist showed a weak modulation of cytokine expression in stimulated whole blood which did not correlate with the inhibition pattern of a ginger Hot Flavor™ extract. Whether ginger constituents have an effect on central 5-HT_{1A} receptors, which would explain the use of ginger against mild mental complaints, remains ambiguous. Sufficiently high concentrations might only be achieved locally; effects on the gastrointestinal tract upon ingestion of ginger preparations seem more likely.

4.4 Effects on Lymphocytes

4.4.1 MAP Kinases

To address the effect of ginger constituents on MAP kinases previously reported with cancer cells, we assessed the effect on ERK, JNK and p38 in primary human T-cells. PMA/αCD3 stimulated lymphocytes were used to determine the modulation of MAP kinases by measuring their phosphorylation state using a commercial CBA assay. Experiments using primary T cells could not support earlier findings [86] that ginger extracts and its main constituents inhibit the MAP kinases p38, ERK1/2, or JNK.

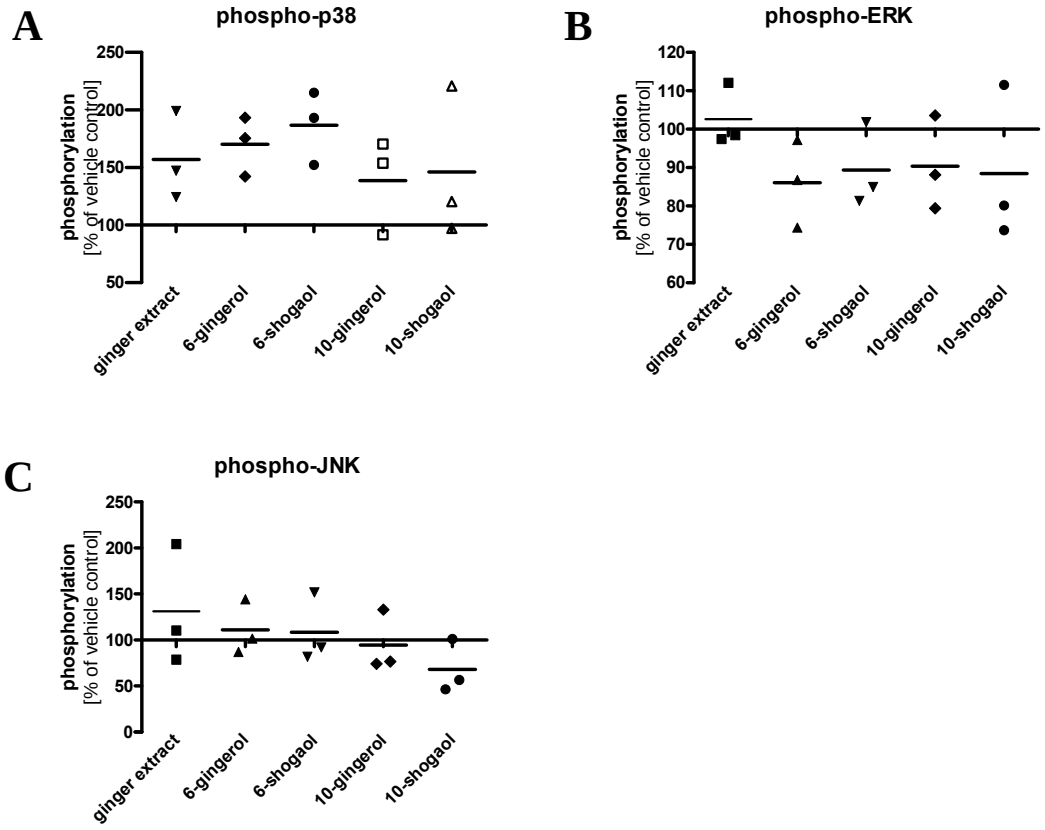


Figure 33: MAP kinase phosphorylation in isolated lymphocytes.

Ginger Hot Flavor™ extract (10 µg/ml) and its main constituents (10 µM) were tested on their ability to influence MAP Kinase phosphorylation in isolated human lymphocytes stimulated with αCD3 and PMA. Phosphorylation levels of p38 (A), ERK (B), and JNK (C) were determined using a cytometric bead array by BD.

Given are the values of three experiments (dots) and their mean value (line) of lymphocytes from three different donors.

Phosphorylation of p38 was tendentially up-regulated by 50 to 100 % (Fig. 33 A). Phosphorylation of ERK1/2 was slightly but non-significantly inhibited by pure gingerol-type compounds (Fig. 33 B). Only 10-shogaol significantly inhibited JNK phosphorylation by approximately 35 %, while the whole extract showed a trend towards activation of this kinase. (Fig. 33 C). These findings have been published [328].

The assay setting for MAP kinase phosphorylation might have been suboptimal. JNK phosphorylation was barely detectable. The low amount of phosphorylated protein in isolated lymphocytes can be explained by a requirement for gene expression in isolated naïve T_H1 and $CD8^+$ cells and by its unresponsiveness to TCR ligation in T_H2 cells compared to high constitutive expression levels in the Jurkat cell line [248]. ERK and p38 phosphorylation was robust but nevertheless at a low level; Clöez-Tayarani et al. [444] cultured their lymphocytes for three days to get detectable levels of ERK phosphorylation with serotonin as stimulus whereas p38 phosphorylation could only be non-significantly induced in fresh lymphocytes.

4.4.2 Proliferation

The αCD3 mediated proliferation of isolated lymphocytes was investigated in the presence of ginger extracts and pure compounds thereof. The ginger Hot Flavor™ extract, but not the ginger totum extract or the ginger essential oil (all at 10 µg/ml) showed an inhibition of proliferation of around 20% (Fig. 34 and 35). Due to the susceptibility of monocytes towards gingerol-like compounds, isolated constituents (6-G, 10-G, 6-S, 10-S, 6-GDO, 10-GDO, and 6-DHP) were screened at only 1 µM; additionally, 6-G, 10-G, and 6-GDO were also tested at 5 µM. Only 10-shogaol showed a minimal inhibitory activity and was screened over a concentration range of 0.5 to 10 µM with a significant inhibition above 5 µM (Fig. 35). These data have been published [328].

Due to huge inter-experimental variability, not the absolute amount of proliferated cells (Fig. 34 A) was used for evaluation but the relative amount (Fig. 34 B) with the vehicle control (αCD3) set as 100 % for each separate experiment.

Furthermore, a clear correlation between proliferation rate and contaminating monocytes could be observed and is in agreement with earlier reports [445, 446].

DPAT and NAN-190 were also tested at 100 nM and 100 µM without any effects, showing that neither serotonin agonism nor antagonism had any influence on proliferation under the assay conditions used (Fig. 36). It could be observed that monocyte contamination strongly

improved proliferation causing visible cell aggregate formation by lymphocyte migration and subsequent proliferation whereas αCD28 co-stimulation had no influence.

Inhibition of proliferation can be regarded as an additional indication for $iPLA_2$ inhibition because proliferation of lymphocytes, Jurkat cells [219], and monocytes [220] is $iPLA_2$ dependent. Furthermore, it is independent of $5\text{-}HT_{1A}R$ ligation.

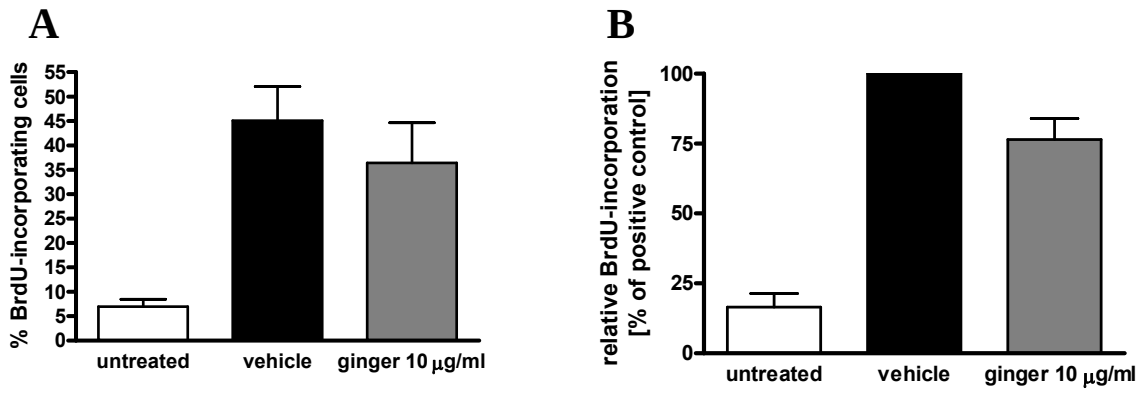


Figure 34: Proliferation of αCD3 stimulated lymphocytes

Isolated lymphocytes were stimulated with αCD3 Σ-chain in the presence of BrdU for five days and then stained with a FITC labelled BrdU antibody and subsequently analized by flow cytometry. The absolute percentage of BrdU incorporating (e.g. proliferating) cells is depicted in A. The positive control (vehicle) exerted a huge inter-experimental variability (between 20 and 75 %) whereas additional ginger Hot Flavor™ extract (ginger) at 10 µg/ml caused a reduced proliferation with a similar variability (12 - 70 %). B shows the same BrdU incorporation as in A but standardised to 100 % for the positive control (vehicle) markedly reducing these donor-dependent deviations.

Given are the mean values (±SEM) of triplicate experiments with lymphocytes of at least 4 different donors.

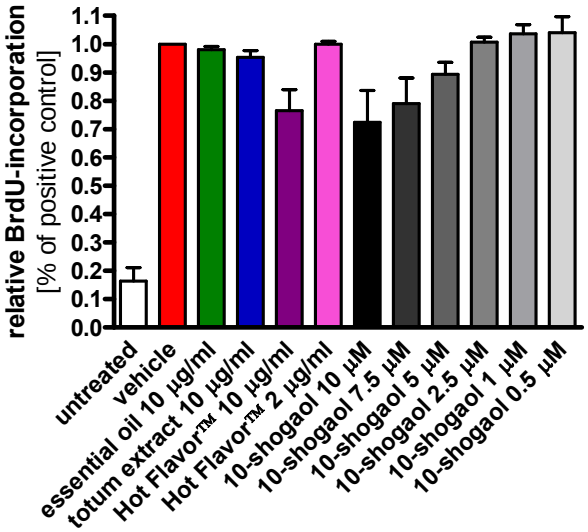


Figure 35: Relative proliferation of αCD3 stimulated human lymphocytes determined by BrdU incorporation.

Ginger Hot Flavor™ extract at 10 µg/ml and 10-shogaol at 5 to 10 µM show a significant inhibition whereas essential ginger oil and the totum extract, and the other main gingerols and shogaols show no effect. The molecular mechanism behind it is unknown but could be an indication for PLA_2 inhibition. Shown is the relative proliferation (+SD) of lymphocytes of at least three different blood donors.

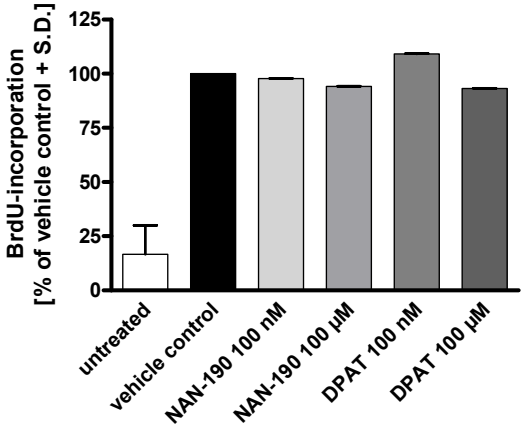


Figure 36: Relative proliferation of αCD3 stimulated human lymphocytes determined by BrdU incorporation.

The selective 5-HT_{1A}R antagonist NAN-190 and the agonist DPAT show no modulation of lymphocyte proliferation. Shown is the relative proliferation (+S.D.) of lymphocytes of three different blood donors.

4.4.3 Calcium in Jurkat Cells

Jurkat cells were chosen as alternative to isolated lymphocytes to determine intracellular calcium liberated from intracellular stores, because they show a strong calcium signal when stimulated with αCD3 (according to Guse et al. released form different calcium stores [447]), and express the 5-HT_{1A}R, which couples to PLC and inhibits adenylat cyclases. Primary human T cells express this receptor only upon activation and its signal transduction is solely via adenylate cyclase [448, 449].

In calcium free medium, the increase in $[Ca^{2+}]_i$ upon αCD3 challenge of Jurkat cells is rapid but transient and falls back to baseline level within two min. Additionally, this cell line exhibited distinct inter-experimental variability .

Ginger extracts showed a weak (less than 15 %) but statistically significant inhibition of antibody mediated intracellular calcium increase (Fig. 37 A). Results from pure compounds show weak inhibitory activities on the CD3 mediated calcium release in Jurkat cells, that are notable only at concentrations minimally below their toxic one. 6-Shogaol seems to be the main compound responsible for the inhibitory activity of ginger extracts. Indeed, the inhibition by the latter can be explained by an additive effect of their main constituents. (Fig. 37 B). The mode of action remains elusive but as neither the extracts nor the pure compounds caused a calcium release in unstimulated Jurkat cells, calcium loss during the incubation period can be excluded as causative action.

Serotonin at 10 nM and 10 µM exerted no modulation of CD3 mediated calcium release but enhanced it by 4 to 15 % at concentrations between 100 nM and 1 µM. NAN-190, a 5-HT_{1A}R antagonists, had no influence most likely due to the absence of endogenous serotonin. DPAT, a 5-HT_{1A}R agonist, showed at 10 nM very inconsistent results in combination with αCD3. Calcium release was up-regulated by 0 to 50 % irrespective of the antibody concentration.

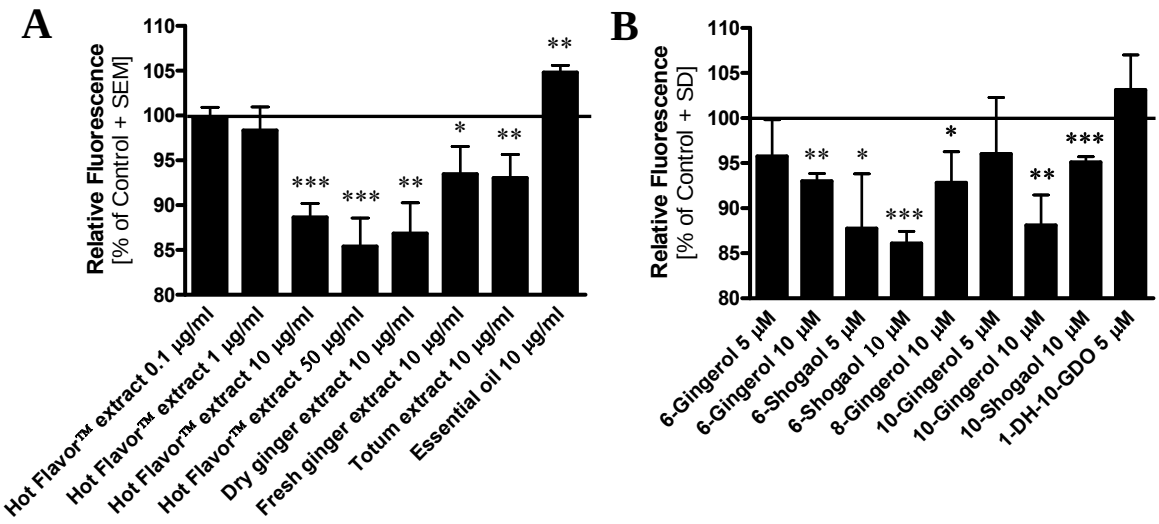


Figure 37: Inhibition of calcium release in stimulated Jurkat cells treated with different ginger preparations.

A The ginger Hot Flavor™ extract showed a dose-dependent inhibition of αCD3 stimulated calcium release from intracellular stores in Jurkat cells. The inhibition was not agonist dependent, because 10 µg/ml ginger Hot Flavor™ extract inhibited 1/10 of the antibody by 93.02 % ± 0.51 (data not shown). Two ethanolic ginger extracts and the totum CO_2 extract showed an inhibition of 7 to 13 % corresponding approximately to their oleoresin content. Ginger essential oil showed a significant but weak amplification of 5 %. **B** Some pure compounds were tested at 5 and/or 10 µM. 6- and 10-gingerol showed a weak inhibition of 7 and 12 %, respectively, only at 10 µM whereas 6-shogaol reduced the calcium response at both ones by 13-14 %. 8-Gingerol at 10 µM was similar to 6-gingerol. 10-Shogaol and 1-dehydro-10-gingerdione (1-DH-10-GDO) were instantaneously cytotoxic at 10 µM and the former showed a weak inhibition of 5 %.

The results of at least three independent measurements are given as percentage of the positive control (calculation see 3.12.1) plus the SEM * $P \leq 0.1$; ** $P \leq 0.05$; *** $P \leq 0.01$ with respect to the vehicle control (100 %).

4.4.4 Conclusions

Our data show no significant effect on MAP kinases, neither on p38 as proposed Kim et al. [86] nor on JNK or ERK tested in PMA/αCD3 stimulated human lymphocytes.

Proliferation of αCD3 stimulated human lymphocytes was maximally reduced by 20% when exposed to 10 μg/ml ginger Hot Flavor™ extract or 10-shogaol up to 10 μM.

Calcium release in αCD3 stimulated Jurkat cells was only reduced by 10 to 15% by 10 and 50 μg/ml ginger Hot Flavor™ extract, respectively. The same inhibition was achieved by 6-shogaol and 10-gingerol both at 10 μM; an additive effect for the extract seems likely.

A physiological relevance for these findings is unlikely. Rather, it is concluded, that ginger extracts and its main constituents exert no anti-inflammatory activity mediated by MAP kinases, lymphocyte proliferation or calcium release.

4.5 Identification of AGPs as TLR-Ligands

4.5.1 *In Vitro* Effects of AGPs

In accordance with the observations of Chang et al. [450] we found that hydro-alcaholic and aqueous ginger preparations induce the expression mainly of IL-6 and -8 in human whole blood whereas ginger extracts free of hydrophilic compounds and macromolecules did not (Fig. 38).

Furthermore, it could be demonstrated that purified AGPs from *Physcomitrella* and radish root as well as aqueous extracts from different vegetables, herbs, and spices induced monokines (TNF-α, IL-6, IL-1β, IL-8, and IL-10) in human whole blood at 50 µg/ml (Table 7). The absence of endotoxin contamination was determined at Cambrex, UK.

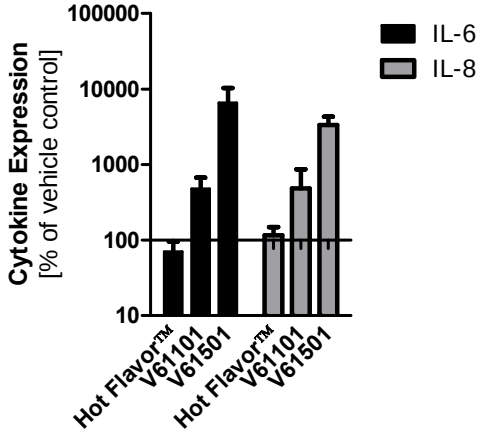


Figure 38: Modulation of the cytokine expression by ginger extracts.

Human whole blood was stimulated with the ginger CO_2 Hot Flavor™ extract and two hydro-alcoholic ginger extracts all at 50 µg/ml. The former was free of hydrophilic constituents whereas the latter to contained proteins and sugars. Cytokine expression was determined after 18 h incubation at 37 °C using the CBA inflammation kit. IL-6 and -8 were upregulated by the latter two extracts (V61101and V61501) whereas the former (Hot Flavor™) exerted no significant modulation. The relative expression of a triplicate screening in % of the vehicle control is given.

Isolated *D. carota*, *D. filix-mas*, *E. arvense*, *T. vulgaris*, and *Z. officinale* AGPs retained partial stimulatory activity whereas the activity of the AGP-deprieved extracts was likely caused by residual Yariv´s reagent, a strong cytokine inducer (Table 7). A screening of IL-1β with 152 aqueous plant extracts of more than 100 families showed that over 80 % of these had a pro-inflammatory activity *in vitro.* Most of these extracts contained AGPs but there was no correlation between AGP content and cytokine induction [451]. At least *in vitro*, hydrophilic plant constituents and in part AGPs induce the expression of cytokines

typical for activated monocytes. Furthermore, Morello at al. demonstrated, that only AGP containing β-1,6 and β-1,3-linked galactans linked to proteins are active and displace fluorescent labeled LPS from its TLR4 binding site [451].

The monocyte-like U-937 cell line was tested for its suitability as alternative to whole blood. Only differentiation of U-937 into MΦ led to an enhanced production of cytokines upon stimulation with LPS and haIgG, respectively. But like whole blood these cells produce inflammatory cytokines upon ethanolic ginger extracts stimulation (Fig. 39).

Therefore, they could not be used but nevertheless gave a hint that the observed cytokine induction by hydro-alcaholic plant preparations is mediated directly by MΦ and other cell types are dispensable.

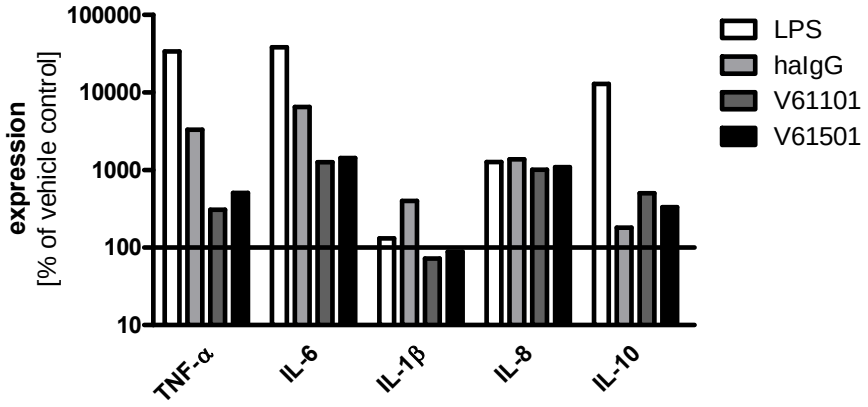


Figure 39: Modulation of the cytokine expression in differentiated U-937 macrophages.

Modulation pattern of LPS, heat-inactivated IgG (haIgG) and two ginger extract (V61101 and V61501) on cytokine expression in PMA differentiated U-937 MΦ after 9 hours incubation.

Shown are the results of a triplicate screening in % of the vehicle control.

	TNF-α pg/ml±SEM	IL-6 pg/ml±SEM	IL-1β pg/ml±SEM	IL-8 pg/ml±SEM	IL-10 pg/ml±SEM
Negativ	50 ± 9	69 ± 20	555 ± 85	507 ± 75	33 ± 6
LPS	11448 ± 1406	15897 ± 1423	8710 ± 1155	24664 ± 3581	4401 ± 621
Physcomitrella AGP	2322 ± 713	9820 ± 1873	273 ± 81	15981 ± 3265	471 ± 134
Radish root AGP	384 ± 191	3087 ± 1531	101 ± 33	8474 ± 3719	29 ± 13
Radish root AGP A-1	304 ± 147	2925 ± 1388	100 ± 25	6609 ± 2594	46 ± 22
Apium graveolens	316 ± 104	4547 ± 1016	180 ± 41	9292 ± 2125	94 ± 27
Agropyron repens	474 ± 175	5064 ± 992	274 ± 25	7430 ± 2313	178 ± 68
Armoracia rusticana	12 ± 5	552 ± 250	128 ± 35	2926 ± 1106	4 ± 1
Alpinia cf. officinale	4138 ± 1121	8576 ± 100	2072 ± 459	11700 ± 1353	1123 ± 163
Brassica oleraceae	8 ± 4	100 ± 36	126 ± 33	980 ± 311	2 ± 1
Beta vulgaris	7 ± 2	337 ± 146	128 ± 44	2206 ± 754	2 ± 1
Cucurbita pepo	47 ± 17	1508 ± 553	162 ± 36	4604 ± 1817	6 ± 1
Daucus carota	4808 ± 949	8644 ± 339	2738 ± 712	15130 ± 3163	2081 ± 495
D. carota w/o AGP	18066 ± 3384	23655 ± 1550	7905 ± 1219	77767 ± 12931	3161 ± 538
D. carota AGP	609 ± 79	11901 ± 1941	1171 ± 86	11625 ± 3615	222 ± 42
Dryopteris filix-mas	1490 ± 538	7833 ± 784	744 ± 193	11010 ± 2300	606 ± 180
D. filix-mas w/o AGP	1658 ± 202	18164 ± 2226	1181 ± 58	22644 ± 4803	291 ± 53
D. filix-mas AGP	83 ± 3	244 ± 57	800 ± 6	1347 ± 246	52 ± 2
Equisetum arvense	1322 ± 511	6722 ± 857	511 ± 122	9639 ± 2028	490 ± 158
E. arvense w/o AGP	14 ± 16	196 ± 1179	38 ± 14	822 ± 2920	5 ± 4
E. arvense AGP	116 ± 11	2282 ± 555	852 ± 23	4707 ± 1134	62 ± 5
Echinacea purpurea	9 ± 3	161 ± 78	169 ± 20	1733 ± 730	2 ± 1
Foeniculum vulgare	501 ± 176	5260 ± 945	241 ± 29	8191 ± 2268	177 ± 68
Ononis spinosa	1823 ± 682	8134 ± 587	715 ± 181	13803 ± 2823	731 ± 186
Raphanus sativus	10 ± 3	345 ± 162	163 ± 44	1441 ± 541	4 ± 2
Thymus vulgaris	1340 ± 568	7206 ± 913	623 ± 158	12516 ± 2646	578 ± 198
T. vulgaris w/o AGP	1400 ± 177	19096 ± 2294	1060 ± 48	53982 ± 11620	313 ± 56
T. vulgaris AGP	123 ± 11	2581 ± 690	809 ± 18	5160 ± 1274	63 ± 5
Zingiber officinale	854 ± 391	4238 ± 1172	377 ± 95	6904 ± 2651	274 ± 132
Z. officinale w/o AGP	71 ± 3	266 ± 64	763 ± 8	4910 ± 1750	51 ± 2
Z. officinale AGP	77 ± 1	101 ± 16	819 ± 20	1389 ± 428	48 ± 1
Yariv's reagent	13505 ± 3155	23239 ± 1578	10939 ± 1606	28331 ± 8842	6053 ± 803

Table 7: Cytokine modulation in whole blood by AGP-rich plant preparations.

Absolute cytokine expression in pg/ml upon treatment of human whole blood with 50 µg/ml aqueous plant extracts for 18 h at 37 °C. Cytokines were determined by CBA and FACS analysis.

AGPs from selected extracts (*D. carota*, *D. filix-mas*, *E. arvense*, *T. vulgaris*, and *Z. officinale*) were isolated as described in Materials and Methods and the AGPs and the AGP-deprived extracts were tested at 50 µg/ml original extract. Due to residual Yariv´s reagent in the latter, this compound was also tested at 50 µg/ml.

Shown are mean values ± SEM of blood samples from three different donors each measured in a triplicate.

4.5.2 Effects of AGPs in Caco-2 Whole Blood Co-culture Assay

On the search for the active principles of aqueous plant extracts causing pro-inflammatory cytokine expression in whole blood we decided, motivated by Eguchi et al. [452], to use the Caco-2 co-culture model to separate small organic compounds from high molecular weight compounds. In theory, only small molecules fulfilling the Lipinski Rule of Five [453] cross the intestinal lining or a Caco-2 monolayer. Therefore, a combined absorption - whole blood assay would limit the active principles in the blood compartment by excluding for example macromolecules and provide knowledge about their bioavailability.

To discriminate between direct effects on blood cells and Caco-2 blood interactions not only tight monolayers with a TEER exceeding 400 Ω/cm^2 but also 'leaky' ones with a low TEER of around 200 Ω/cm^2 were used.

Whole blood treated with ethanolic ginger extracts (50 µg/ml) showed a significant cytokine induction likely due to contaminating AGPs. Therefore, one such extract was tested in the Caco-2 whole blood co-culture at 50 µg/ml apical concentration. The corresponding experiments showed high variability, but no statistically significant modulation of apical and basolateral cytokine expression on tight and leaky monolayers (Fig. 40 A and 41 A). The aim of eliminating pro-inflammatory activities was not achieved with respect to ethanolic ginger extracts. This might by either due to resorbable pro-inflammatory constituents or to anti-inflammatory activities on Caco-2 cells inhibiting their formation of PGE_2 [454]. The latter might cause a reduction in the observed anti-inflammatory effect on leukocytes [417] leading finally to a cytokine induction (see chapter 4.2.3).

In contrast an aqueous carrot extract strongly induced cytokine expression in whole blood; these results were consistent and significant. Cytokine expression in the basolateral blood compartment was completely obviated by apical application of the extract to a tight Caco-2 monolayer (Fig. 40 B). On the other hand, a leaky monolayer only partially prevented cytokine induction in the latter setting (Fig. 41 B). This finding points toward a high-molecular weight active principle which is likely to be non-absorbable *in vivo*. The ability of the carrot extract to induce cytokine production on the apical side is likely related to an effect on Caco-2 cells and does not affect basolateral measurements. It can further be concluded that the co-culture assay is a useful tool when effects in the basolateral compartment are strong enough so as to outweigh experimental variability.

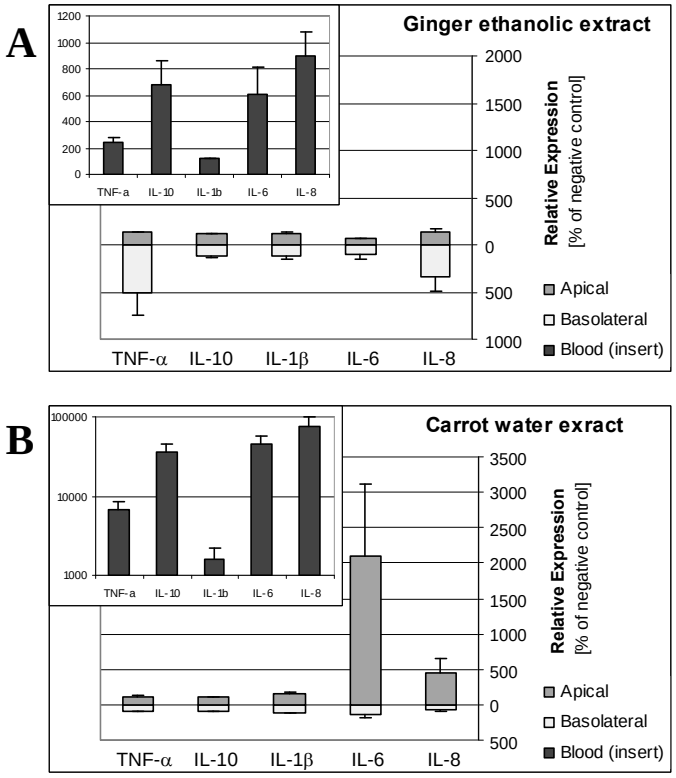


Figure 40: Cytokine expression in the Caco-2 co-culture (TEER >400 Ω/cm^2) and in whole blood.

An ethanolic ginger extract or an aqueous carrot extract were applied to the apical side of a Caco-2 co-cultures (at 50 µg/ml) and cytokine expression was determined on both sides after 18 hours of incubation. The two extracts were also added to the basolateral compartment at a theoretical equilibrium concentration of 10 µg/ml (inserts). **A** The ginger extract added to the apical side caused nearly no change of cytokine baseline levels. Only in one of three cases basolateral expression of TNF-α and IL-8 was up-regulated. Addition to the basolateral side resulted in a moderate but significant induction of IL-10, IL-6, and IL-8. **B** In contrast, carrot extract applied apically stimulated IL-6 and IL-8 on the apical side but with high variability and without statistical significance. Basolateral cytokine expression was unaffected. Carrot extract added to the basolateral compartment resulted in a strong induction of TNF-α, IL-10, IL-1β, IL-6, and IL-8.

Given are the mean values + SEM of three independent experiments (three different blood donors and three different Caco-2 passages) each done in a duplicate. Y-values are in % of the corresponding vehicle controls. Note the logarithmic scale of insert B.

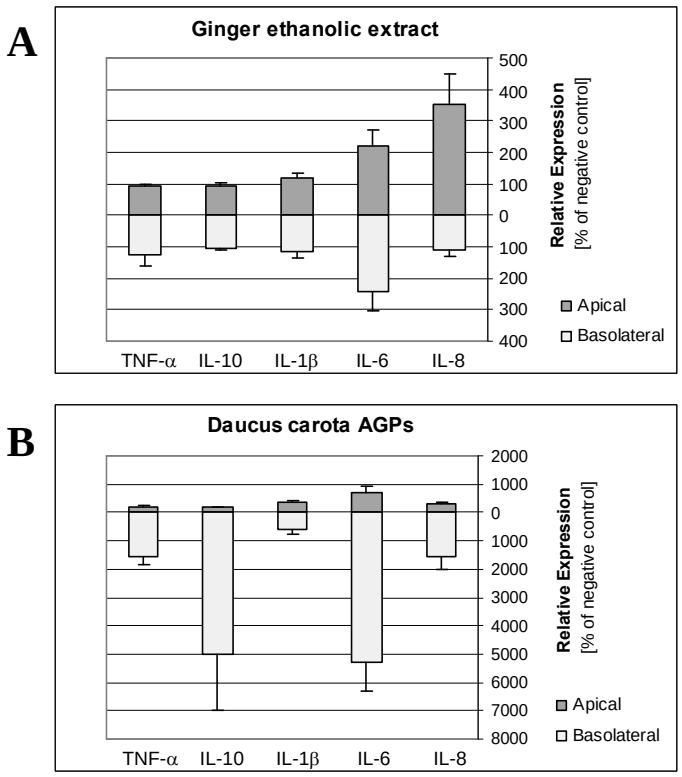


Figure 41: Cytokine expression in the Caco-2 co-culture (TEER ~200 Ω/cm^2).

The same experiments as in Fig. 39 were done using Caco-2 membranes with a TEER of around 200 Ω/cm^2, thus allowing partial para-cellular diffusion.

An ethanolic ginger extract or an aqueous carrot extract were applied to the apical side of a Caco-2 co-culture (50 µg/ml) and cytokine expression was determined on both sides after 18 hours of incubation. (A) When incubated with the ginger extract, cytokine expression was only insignificantly changed. (B) In contrast, TNF-α, IL-10, IL-1β, IL-6, and IL-8 were strongly and significantly up-regulated on the basolateral side of the carrot treated co-cultures. The moderate induction on the apical side is of no statistical significance.

Given are the mean values + SEM of three independent experiments (three different blood donors and three different Caco-2 passages) each done in a duplicate. Y-values are in % of the corresponding vehicle controls.

4.5.3 Effects of AGPs in TLR Knock-Out Mouse Blood

In fresh whole blood from wild-type mice (diluted 1:1 with HBSS) lipopolysaccharide, zymosan A, and *Daucus carota* AGP caused a significant expression of TNF-α (Fig. 42 A) and IL-6 (Fig. 42 B) after 18 hours, whereas IL-10 (Fig. 42 C) was only induced by zymosan A. IL-12p70, IFN-γ, and MCP-1 were not expressed (data not shown).

Blood from $TLR4^{d/d}$ mice showed a less pronounced TNF-α and IL-6 expression upon LPS and AGP challenges, whereas there was no alteration with zymosan A. Blood from $CD14^{-/-}$ mice showed a similar, but less pronounced reduction and cytokine expression by zymosan A was unaffected.

LPS and AGP were unable to induce cytokines in blood from $MyD88^{-/-}$ mice, whereas zymosan A lead to markedly reduced TNF-α expression, reduced expression of IL-6, and no IL-10 induction.

These results show that, at least in mice, APGs i) share the same TLR4- and MyD88 dependent pathways as LPS, ii) bind to CD14, and iii) have no other signalling pathway coupled to the induction of TNF-α, IL-6, and IL-10.

The partial induction of cytokines by LPS in $TLR4^{d/d}$ mouse blood might be mediated by contaminants, but still is MyD88 dependent. As expected, the TLR2 ligand zymosan A signals through MyD88 dependent and independent pathways.

Notably, inter-individual variability was much less pronounced in murine blood than in human.

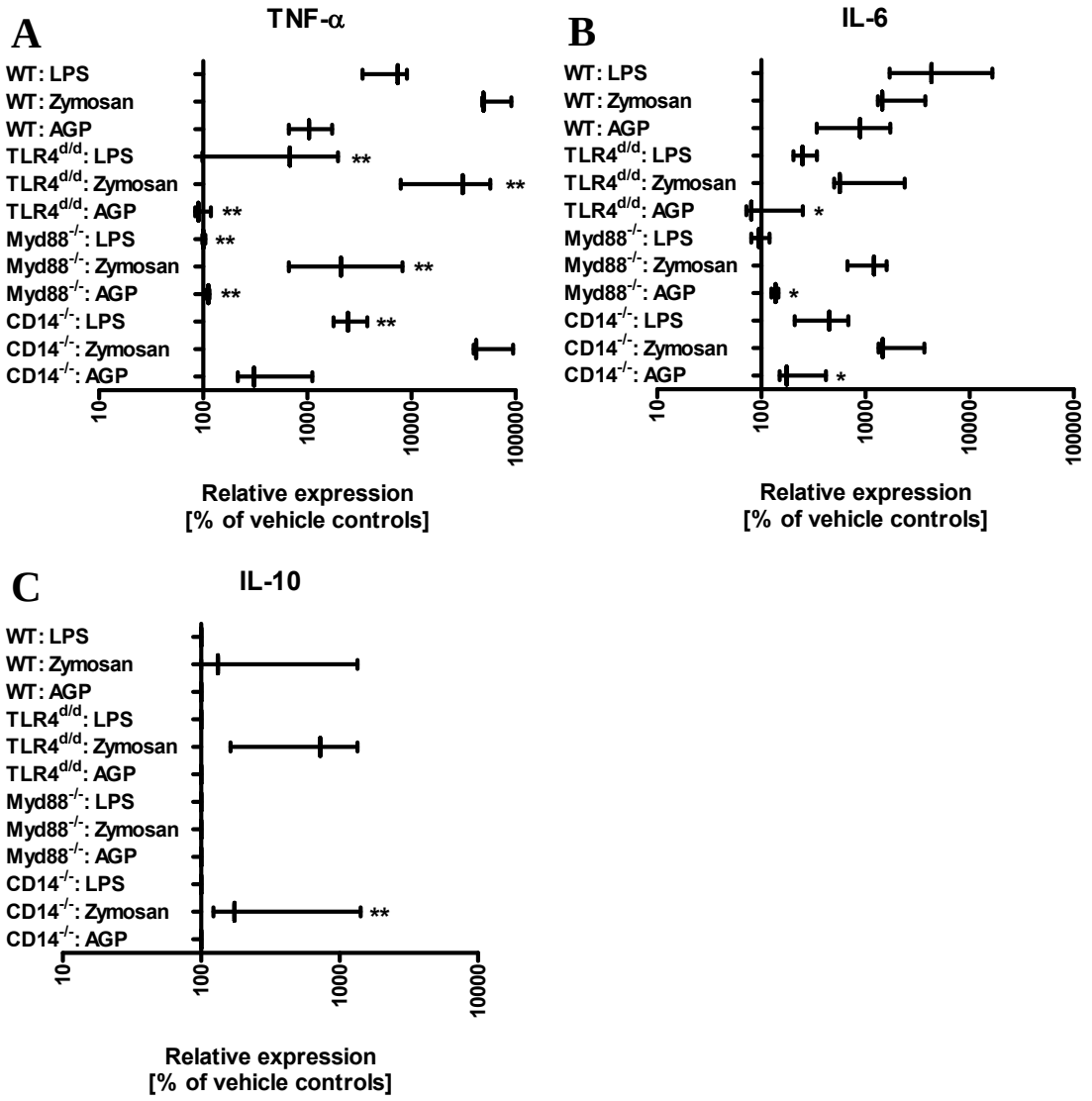


Figure 42: Cytokine expression in mouse blood.

Fresh blood of wild type, TLR4$^{d/d}$, Myd88$^{-/-}$, and CD14$^{-/-}$ mice diluted 1:1 with HBSS was stimulated for 18 hours with a vehicle control, lipopolysaccharide (LPS), zymosan A, and *D. carota* AGP (AGP), respectively. Of all measured cytokines **A** TNF-α, **B** IL-6, and **C** IL-10 were significantly induced by at least one stimulus. Cytokine expression induced by LPS is facilitated by CD14, is mainly mediated by TLR4 and dependent on Myd88. AGP signalling is strictly TLR4 and Myd88 dependent and also facilitated by CD14. Zymosan A signalling is TLR4 and CD14 independent and involves in part Myd88.

Box-and-whisker plot of three independent experiments are given as percent of the corresponding vehicle control.

4.5.4 *In Vivo* Effects of AGPs in Mice

Purified AGPs from *Daucus carrot* potently induced inflammation mediators (NO, IL-1β, and TNF-α) in mice upon peritoneal injection and caused edema formation upon intraepidermal injection comparable to positive controls (LPS, carrageenan, and zymosan A). NO was induced 5-fold by AGPs (1 mg / 100 g), 2-fold by LPS (250 µg / 100 g), and 3-fold by zymosan A (1 mg / 100 g), respectively. IL-1β expression was induced 2-fold by AGPs and zymosan A and 3.5-fold by LPS, whereas TNF-α expression was induced 1.6-fold by AGPs, 1.7-fold by zymosan A and 1.3-fold by LPS, respectively. Edema formation was measured over time and showed similar kinetics for APGs and saline, with a maximum reached after 60 min; edema volume was twice as high for APGs as for saline (Fig. 43). Carrageenan led to faster edema formation with a maximum before 30 min and the same volume as for AGPs at 60 and 90 min, whereas zymosan A showed the same edema volumes as AGPs up to 60 min, but with volumes still increasing up to at least 90 min.

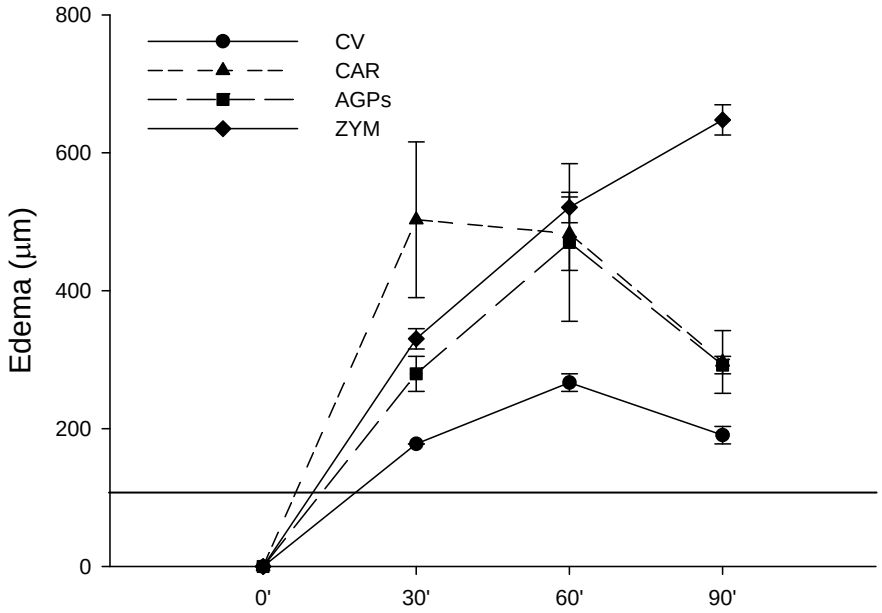


Figure 43: Mouse paw edema induction by arabinogalactan-proteins.
Mice were treated with 20 µl intraepidermal injection of a saline solution (CV), or 0.2% solutions of carrageenan (CAR), *D. carota* AGP (AGPs), or zymosan A (ZYM). Shown are the mean values (edema diameter in µm) of three independent experiments ± SD.

These results clearly show that the ubiquitous AGPs may exert pro-inflammatory activities when administered by circumventing oral application. They emphasize he necessity of appropriate assay strategies for the investigation of aqueous plant extracts. Furthermore, they support the *in vitro* data and put the immunomodulatory activities of many medicinal plants into question.

4.5.5 Conclusions

Arabinogalactan-proteins are an integral part of higher plants and likely a contaminant in many aqueous extracts tested *in vitro* and *in vivo*. We could demonstrate that AGPs are easily extracted from a manyfold of different plant genera, that they elicit strong cytokine expression in whole blood and monocytes, and that they bind to the LPS binding site of TLR4. As expected, AGPs were not resorbed in a combined resorpion/whole blood assay. AGPs are a part of our daily nutrition but when applied invasively (e.g. intraepithelial, intraperitoneal) they lead to a fast increase in plasma TNF-α, IL-1β, and NO; a clear indication for a pro-inflammatory reaction. We conclude that AGPs are responsible for what has been called the “immunostimulatory” or immunomodulatory activity of many medicinal plants, but that this effect is ubiquitous and of no physiologic relevance, as AGPs are not bioavailable.

5. Conclusions and Outlook

We established a whole blood assay suitable for the screening of pro- and anti-inflammatory activities of drugs and multi-component mixtures (e.g. plant extracts) which led to the finding that ginger rhizome (50µg/ml) and its individual phenylpropanoid constituents (50 µM) exhibit *in vitro* anti-inflammatory activities mainly by inhibiting IL-1β expression, independent of the stimulus used. By means of a newly developed fluorescence coupled mixed micelle-based PLA_2 assay, standard SDS-PAGE and Western blot, and FACS analysis we have demonstrated potent inhibition of cytosolic and calcium-independent phospholipases A_2-dependant IL-1β maturation and secretion, whereas negligible effects were found on IL-1β transcription and translation, distinct ion fluxes, or MAP kinase inhibition. IL-1β expression, i-/cPLA_2 activities, and PGE_2 production are markedly inhibited by 2-10 µg/ml ginger extracts and 2-10 µM of isolated constituents; these concentrations are below those that have been reported previously for COX and LOX inhibition [80-82]. Therefore, we conclude that the i-/cPLA_2 inhibition, which deprives COX isoenzymes of their substrate, is most likely the basis not only for the well documented inhibition of PGE_2 expression in monocytes, but also plays a dominant role in the treatment of inflammatory complaints. Surprisingly, ginger extract and 10-shogaol led to an increase in total intra- and extracellular free arachidonic acid. This seems to be due to a differential inhibition of distinct PLA_2 isoforms, a concomitant inhibition of iPLA_2 and COX/LOX enzymes, and maybe other, yet undetermined and spatially separated effects. The absorption and cell membrane affinity of the main ginger constituents determined in a Caco-2 assay, underpinned by recent pharmacokinetic data [55-59], suggest that ginger constituents likely reach sufficiently high concentrations for the in vivo manifestation of the above effects only when applied topically (dermatica) or in the gastrointestinal mucosa and may not be effective for a systemic use. Notably, the physiological relevance of the observed effects has to be confirmed *in vivo*.

Bioactivity-guided isolation of constituents of a ginger extract revealed that several phenylpropanoids (notably not the main constituent 6-gingerol) have an IC_{50} of 3 to 20 µM at the 5-HT_{1A}R. Ginger extract acts as a partial agonist with a 60 % of the maximal receptor activation at 25 µg/ml, compared to the maximal activation by the full agonist DPAT. The isolated compounds exert either no activation or act as weak partial agonists (10-gingerol, 10-shogaol, and 1-dehydro-6-gingerdione). Ginger is used in traditional medicine against mental disharmony and to alleviate the well-being; at the same time the 5-HT_{1A}R is

involved in anxiety. However, a sufficiently high CNS concentration of ginger constituents is barely achieved and this connection remains speculative. Because effects of ginger extracts and isolated constituents on cytokine-expression in whole blood, lymphocyte proliferation, and calcium-release in Jurkat cells could not be linked to the 5-HT_{1A} receptor, no conclusion regarding serotonergic anti-inflammatory effects can be drawn and this question has to await further examinations.

Finally, we could demonstrate in human whole blood, isolated monocytes, and blood of TLR4 knock-out mice that ubiquitous plant arabinogalactan-proteins exert a strong pro-inflammatory activity by CD14 mediated TLR4 binding and subsequent MyD88-dependent induction of monokine expression. Additionally, *in vivo* experiments with purified *D. carota* AGPs in mice showed strong edema formation and pro-inflammatory cytokine and NO induction upon intraepithelial and intraperitoneal administration, respectively. A combination of the whole blood absorption assay confirmed the expectation that the high-molecular weight AGPs are likely to be non-absorbable. These findings show that AGP contamination is likely responsible for several observed *in vitro* immunomodulatory or even "immunostimulatory" effects of medical plants and that these are artificial and of no physiological relevance. Therefore, reports on medical plants claimed to be immunomodulatory have to be treated with care.

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