Pharmacologic Investigation and Identification of Molecular Modes of Action of Defined Extracts and Components from Frankincense

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Pharmakologische Untersuchung und Identifizierung von Wirkmechanismen definierter Extrakte und Komponenten des Weihrauchharzes

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

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2012

Tag der mündlichen Qualifikation:	26.07.2012
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1 Abbreviations

5-H(P)ETE	5(S)-hydro(pero)xy-6-trans-8,11,14-cis-eicosatetraenoic acid
5(S),12(S)-diHETE	5(S),12(S)-dihydroxy-6,10-trans-8,14-cis-eicosatetraenoic acid
3α-Ac-7,24-dien-TA	3α-*O*-acetyl-7,24-dien-tirucallic acid
(3α-)Ac-(8,24-dien-)TA	3α-*O*-acetyl-8,24-dien-tirucallic acid
3α-OH-(7,24-dien-)TA	3-α-hydroxy-7,24-dien-tirucallic acid
3α-OH-8,24-dien-TA	3-α-hydroxy-8,24-dien-tirucallic acid
3β-OH-(8,24-dien-)TA	3-β-hydroxy-8,24-dien-tirucallic acid
3-oxo-TA	3-oxo-8,24-dien-tirucallic acid
12-H(P)ETE	12(S)-hydro(pero)xy-10-trans-5,8,14-cis-eicosatetraenoic acid
12-HHT	12(S)-hydroxy-5-cis-8,10-trans-heptadecatrienoic acid
15-H(P)ETE	15(S)-hydro(pero)xy-13-trans-5,8,11-cis-eicosatetraenoic acid
AA	arachidonic acid
A-α-BA	3-*O*-acetyl-α-boswellic acid
A-BA	3-*O*-acetyl-β-boswellic acid
a. f.	acid fraction
Ac-LA	3-*O*-acetyl-lupeolic acid
Ac-OH-LA	3-*O*-acetyl-28-hydroxy-lupeolic acid
AKBA	3-*O*-acetyl-11-keto-β-boswellic acid
ATP	adenosine triphosphate
(β-)BA	(β-) boswellic acid
BAPTA-AM	1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester)
BSA	bovine serum albumin
C1P	ceramide-1-phosphate
Ca^{2+}	calcium (ions)
CaMKII	Ca^{2+}/calmodulin-modulated protein kinase II
cAMP	cyclic adenosine monophosphate
CDC	cinnamyl-3,4-dihydroxy-α-cyanocinnamate
CG	cathepsin G
COX	cyclooxygenase
$cPLA_2$	cytosolic phospholipase A_2
CysLT s	cysteinyl-leukotrienes

DH-NA	4(23)-dihydro-nyctanthic acid
DH-(k-)RA	4(23)-dihydro-(11-keto-)roburic acid
DMSO	dimethylsulphoxide
EDC	N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
EDTA	ethylenediaminetetraacetate
ERK	extracellular signal-regulated kinase
FAF-BSA	essentially fatty acid-free bovine serum albumin
FCS	foetal calf serum
FLAP	5-LO activating protein
fMLP	formyl-methionyl-leucyl-phenylalanine
Fura-2-AM	1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methyl-phenoxy) ethane-N,N,N',N'-tetraacetic acid, pentaacetoxy-methyl ester
GFP	green fluorescent protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HLE	human leukocyte elastase
HPLC	high performance liquid chromatography
IFNγ	interferon-γ
Ig	immunoglobulin
IKK	IκB kinase
IL	interleukin
iNOS	inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
KBA	11-keto-β-boswellic acid
LA	lupeolic acid
LO	lipoxygenase
LPS	lipopolysaccharide
LT	leukotriene
MAPK	mitogen activated protein kinase
MEK	MAPK/ERK kinase, MAPK kinase
MMP	matrix metalloproteinase
MNK1	mitogen-activated protein kinase interacting kinase 1
MS	mass spectrometry
MSK1	mitogen- and stress-activated protein kinase 1

(m)PGES(-1)	(microsomal) PGE_2 synthase (-1)
NA	nyctanthic acids
n. f.	neutral fraction
NF-κB	nuclear factor κ B
NMR	nuclear magnetic resonance
PAF	platelet activating factor
PAGE	polyacrylamide gel electrophoresis
PAPC	1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine
PAPE	1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PG	prostaglandin
PG buffer	PBS + glucose (1 mg/ml)
PGC buffer	PBS + glucose (1 mg/ml) + $CaCl_2$ (1 mM)
PIP	polyphosphoinositide
PLA_2	phospholipase A_2
PMA	phorbol myristate acetate
PMNL	polymorphonuclear leukocytes
PMSF	phenylmethylsulphonylfluoride
POG	1-palmitoyl-2-oleoyl-*sn*-glycerol
PPAR	peroxisomal proliferator-activated receptor
PRAK1	p38-regulated/activated protein kinase 1
RA	roburic acid
RE	raw extract
RT	room temperature
SDS	sodium dodecyl sulphate
STAT	signal transducer and activator of transcription
STI	soybean trypsin inhibitor
TA	tirucallic acid
TNFα	tumour necrosis factor α
TX	thromboxane

2 Introduction

2.1 Inflammation

2.1.1 General remarks

Inflammation is a pathophysiological reaction characterized by five cardinal signs: *Rubor*, the reddening of the skin; *calor*, a local warming of the affected area as result of an upregulated metabolic rate; *tumor*, the swelling of the tissue; *dolor*, the painful consequence of swelling and the generation of diverse mediators; and *functio laesa*, the loss of function of the affected organ. Acute inflammation results from stimuli that can be either external or internal. Mechanical stress (through injury or foreign bodies), physical factors (UV or ionizing radiation) and chemical or biological pollutants (acids, bases, heavy metals, bacterial toxins or allergens) represent external stimuli. Internal triggers are tumours, excessive levels of metabolites and physiologic mediators that are released upon stimulation by external stimuli or upon inappropriate regulation of the synthesizing enzymes [1].

2.1.2 Inflammation: A feature of general immune response

Inflammation is the local emergence of an activated immune response that does not only include the tissue that is directly affected but also organs that are widespread in the whole body. The onset and regulation of the inflammatory reaction is a complex mechanism involving a multitude of cell types and can be classified in an innate and an adaptive component. Innate immunity is mainly mediated by phagocytes, natural killer cells and the complement system, which immediately respond to the stimulus. Macrophages are phagocytes that reside in the periphery, primarily in tissues that constantly get in contact with external influences. They recognize common conserved structures of bacteria (e.g. lipopolysaccharide, LPS) by means of surface receptors (toll-like receptors), followed by engulfment of the invader and secretion of cytokines, chemokines, tissue hormones (histamine and serotonin) and lipid mediators (e.g., prostaglandins or leukotrienes) [2]. All these mediators trigger the inflammatory onset in the infected tissue, leading to the cardinal symptoms and preparing optimal conditions for the functioning of successive events of the immune response. Neutrophil granulocytes make up the largest population of leukocytes in the blood (about 60%) and are the main cell type infiltrating the inflamed tissue at the onset of inflammation [1]. Like macrophages, they recognize structures of the bacterial surface and are able to neutralize large numbers of bacteria by phagocytosis. The absorbed bacteria are killed and

degraded in phagolysosomes by superoxide radicals, antimicrobial peptides (defensins) and serine proteases (cathepsin G, human leukocyte elastase and proteinase 3) [3].
The regulation of adaptive immunity is even more sophisticated and more time-consuming. It comprises the specific combat of antigen structures by differentiated lymphocytes, which are able to kill infected cells or to neutralize antigens by means of specific antibodies [1].
Collectively, all these mechanisms are highly regulated by intercellular communication through mediators such as interleukins, prostaglandins and leukotrienes, which represent potential targets of pharmacological intervention.

2.1.3 Chronic inflammation

An efficient immune response represents an essential tool to combat environmental assaults. Nevertheless, the complex immune system is vulnerable to dysregulation. Especially when it is not able to neutralize the stimulating noxa (for example in asbestosis or chronic hepatitis) or the body reacts hypersensitive in response to innocuous and omnipresent antigens (for example in bronchial asthma), the resolution of inflammation cannot be induced and the reaction perpetuates, resulting in chronic inflammation.
Autoimmune disorders represent special chronic inflammatory diseases, characterized by an immune reaction to host antigens, i.e. to structures that are natural part of the body. Here, the immune response leads to persisting inflammation without effectual elimination of the antigen or to a loss of physiologic structures in case of successful elimination. Examples for autoimmune diseases are rheumatoid arthritis, type I diabetes mellitus and multiple sclerosis [1].
Chronic inflammation can lead to severe damage of tissues, to loss of function of the affected organs or even to malign degeneration. Thus, when elimination of an inflammatory noxa is not possible, because it is a self antigen or the immune system is not able to face it, surgery or an anti-inflammatory therapy are rational means to avoid long-term consequences [1, 4].

2.2 Arachidonic acid signalling

2.2.1 Arachidonic acid metabolism and the pathophysiological role of prostaglandins and leukotrienes

Arachidonic acid (AA) is a polyunsaturated fatty acid that is stored in the *sn*-2 position of phospholipids of cellular membranes and is liberated by hydrolysis through phospholipase A_2 (PLA_2) [5]. AA serves as precursor of different groups of lipid mediators (prostaglandins,

thromboxanes and leukotrienes) collectively called eicosanoids, which critically control multiple events of the immune response. The types and proportions of eicosanoids that are produced depend on the enzymatic configuration of the specific cell type and the nature of the stimulus [6]. Naturally, AA supply through phospholipases A_2 plays a central role in the activity of downstream enzymes (Figure **1** and Figure **2**).

Cyclooxygenases (COX) catalyze the first two steps in the synthesis of prostaglandins (PG) and thromboxanes (TX) and produce the unstable hydroperoxide prostaglandin G_2 (PGG_2) and its corresponding alcohol prostaglandin H_2 (PGH_2). PGH_2 is a highly reactive intermediate and is further metabolized dependent on the availability of the respective synthases to thromboxane A_2 (TXA_2), prostaglandin D_2 (PGD_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin E_2 (PGE_2) or prostaglandin I_2 (PGI_2) [7] (Figure **1**). PGs predominantly exert their functions by activation of G protein-coupled receptors: PGD_2 binds to DP1 and DP2 receptors, $PGF_{2\alpha}$, PGI_2 and TXA_2 to FP, IP and TPα/β receptors, respectively, and for PGE_2 even four receptor subtypes (EP1-4) are pharmacologically defined [7].

PGs are produced in a multitude of cells and tissues. In an inflammatory context, granulocytes, macrophages and endothelial cells in the peripheral tissue represent the most important sources leading to inflammatory symptoms and the expansion of the immune response. PGE_2 is produced from PGH_2 by different PGE_2 synthases (see chapter 2.2.4) and plays a predominant role in oedema formation, pain sensitization and fever. In combination wit PGI_2 and PGD_2, PGE_2 dilates blood vessels and thus participates in oedema formation [8]. Pain sensitisation is mainly driven by PGE_2 and PGI_2, which sensitize nociceptors in the periphery [9-10]. PGE_2 not only elicits acute pain, it also enhances inflammatory hyperalgesia and along with PGD_2 augments the processing of pain in the spinal cord (allodynia) [11-12]. Furthermore, PGE_2 is a principal mediator of fever: Stimulation with cytokines that are liberated *inter alia* from stimulated macrophages is supposed to induce the microsomal PGE_2 synthase-1 (mPGES-1) in central nervous structures, which subsequently produces PGE_2 [13-14]. In the thermoregulatory centre in the hypothalamus, PGE_2 causes an increase of the set point of the body temperature and thereby gives rise to the generation of fever [14].

Prostaglandin I_2 (PGI_2, prostacyclin) is produced by PGI_2 synthase and promotes vasodilatation and anti-aggregatory effects in platelets [7]. Within minutes, non-enzymatic hydrolysis of PGI_2 leads to the inactive metabolite 6-keto $PGF_{1\alpha}$, which is commonly used for monitoring of COX or prostacyclin synthase activity [15]. Thromboxane A_2 (TXA_2) is formed from PGH_2 by TXA_2 synthase and leads to artery constriction and platelet aggregation. It is inactivated within a half-life period of about 30 sec by non-enzymatic hydrolysis to thromboxane B_2 (TXB_2) [10]. TXA_2 is the

functional antagonist of PGI_2 and unbalanced thromboxane and PGI_2 levels are supposed to be implicated in the cardiovascular side effects of selective COX-2 inhibitors [7, 16]. Another metabolite of thromboxane synthase is 12(S)-hydroxy-5-cis-8,10-trans-heptadecatrienoic acid (12-HHT) [17], which is also produced spontaneously or catalyzed by ferrous ions, haeme or cytochrome P450 enzymes [18-20]. Its synthesis is enhanced at the expense of PGs in the presence of high glutathione levels [21]. As an affine ligand of the leukotriene B_4 (LTB_4) receptor BLT_2, it induces chemotaxis of mast cells [22]. 12-HHT can be quantified spectrometrically, which makes it a useful marker of COX activity.

Leukotrienes (LTs) are produced from AA by the 5-lipoxygenase (5-LO) in several myeloid cells [23]. This enzyme introduces molecular oxygen into AA leading to 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HPETE) and also catalyzes the subsequent reaction to the epoxide leukotriene A_4 (LTA_4). Alternatively, the unstable hydroperoxide 5-HPETE decomposes to the corresponding alcohol 5(S)-hydroxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HETE) [24]. LTA_4 can either be converted to LTB_4 by LTA_4 hydrolase in neutrophils and monocytes, or to leukotriene C_4 (LTC_4) under coupling of glutathione by LTC_4 synthase in mast cells, macrophages, basophils and eosinophils [25]. Under successive hydrolysis of the peptide bonds, LTC_4 is converted to leukotriene D_4 (LTD_4) and leukotriene E_4 (LTE_4), collectively referred to as cysteinyl-leukotrienes (CysLTs). LTB_4 binds to G protein-coupled receptors; the high affinity type BLT1 and the low affinity type BLT2 [26], but it is also a ligand of the peroxisomal proliferator-activated receptor α (PPARα) [27]. CysLTs bind to the G protein-coupled CysLT1 and CysLT2 receptors [28].

LTs can act as potent inflammatory mediators. LTB_4 modulates the immune reaction as it attracts granulocytes to the inflammatory focus [29]. It increases leukocyte rolling, adhesion on venular endothelia and acts as a chemokine [30-31]. It also leads to enhanced degranulation of leukocytes resulting in the release of reactive oxygen species and lysosomal enzymes [32]. Furthermore, it attracts interleukin-5 (IL-5)-stimulated eosinophils [33] and stimulates the production of IL-5 in T lymphocytes [34]. LTB_4 stimulates the differentiation of B lymphocytes leading to enhanced expression of immunoglobulin E (IgE) receptors and secretion of immunoglobulins [35-36]. Being a ligand of PPARα, LTB_4 is also implicated in transcriptional signalling which possibly leads to anti-inflammatory effects [27]. In monocytes, LTB_4 activates the expression of the pyrogen IL-6 [37]. Moreover, LTB_4 was reported to exhibit hyperalgesic effects [38]. CysLTs are predominantly known for the effective elicitation of brochoconstriction, mucus secretion and vascular leakage combined with enhanced recruitment of eosinophils [39-43]. These airway responses are mostly mediated by CysLT1 receptors. CysLT2-dependent signalling was observed in vascular smooth

muscle cells [44-45]. LTs also play a role in atherosclerosis [46]. Atherosclerotic plaques are commonly infiltrated by leukocytes that produce LTs and thereby sustain inflammatory conditions. 5-HETE and its 5-oxo-metabolite are implicated in chemotaxis and cell proliferation [47-50].

Further lipoxygenases were identified in humans; namely two 12-lipoxygenase isoforms (12-LO, platelet- and epidermis-type), two 15-lipoxygenase isoenzymes (15-LO, leukocyte- and epidermis-type) and the epidermis-type LO 3 [51-53]. 12- and 15-LO produce 12- and 15-HETE, respectively, and in combination with 5-LO lead to the production of lipoxins [51, 54]. 12-HETE was reported to enhance cell proliferation in pancreatic cancer cells [55] and to stimulate cellular mobility of melanoma cells [56]; it is responsible for renal angiotensin II-induced vasoconstriction [57] and acts pro-inflammatory on vascular endothelia [58]. 15-HETE levels are elevated in inflammation but different reports suggest an anti-inflammatory effect that limits an inflammatory overshoot [59]. Lipoxins are anti-inflammatory mediators implicated in the resolution of inflammation [54].

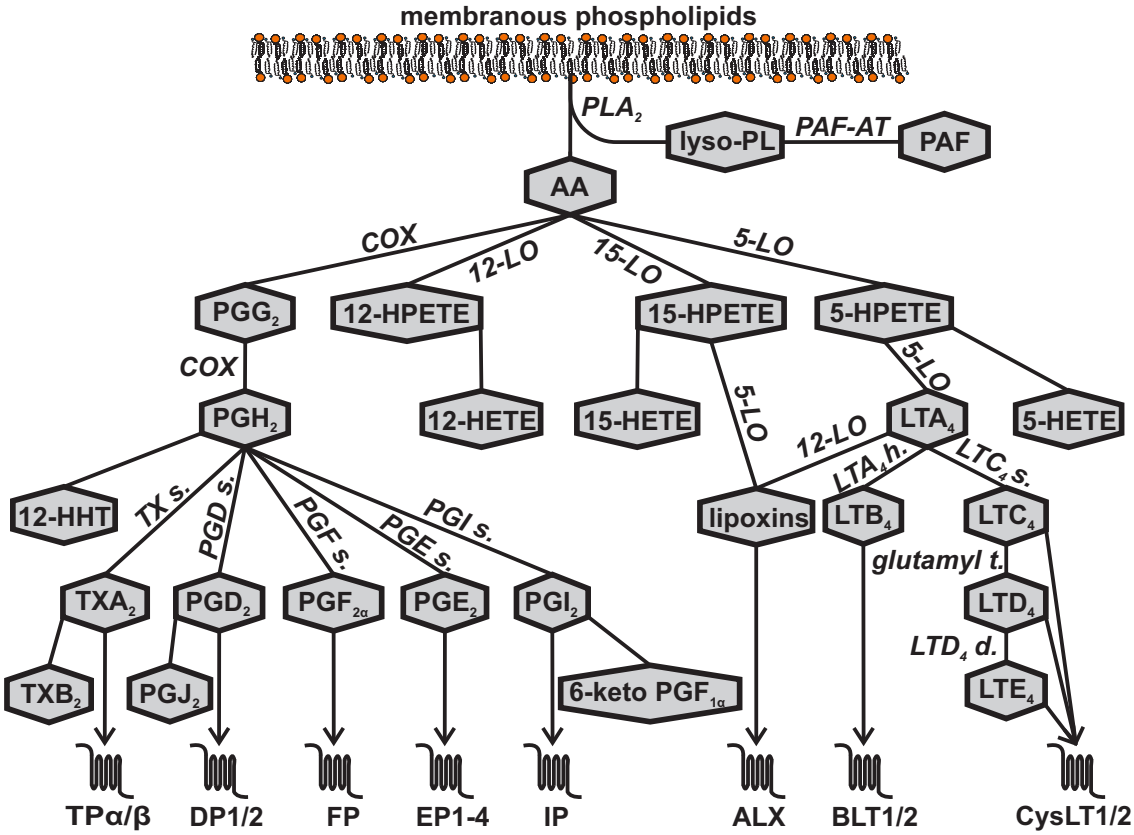


Figure 1: Arachidonic acid signalling. Lysophospholipid (lyso-PL), platelet activating factor (PAF), arachidonic acid (AA), prostaglandin (PG), leukotriene (LT), thromboxane (TX), 12(S)-hydroxy-5-cis-8,10-trans-heptadecatrienoic acid (12-HHT), 5(S)-/12(S)-/15(S)-hydro(pero)xy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-/12-/15-H(P)ETE), cyclooxygenase (COX), lipoxygenase (LO), synthase (s.), LTA_4 hydrolase (LTA_4 h.), γ-glutamyl transpeptidase (glutamyl t.), LTD_4 dipeptidase (LTD_4 d.), PAF acetyl transferase (PAF-AT).

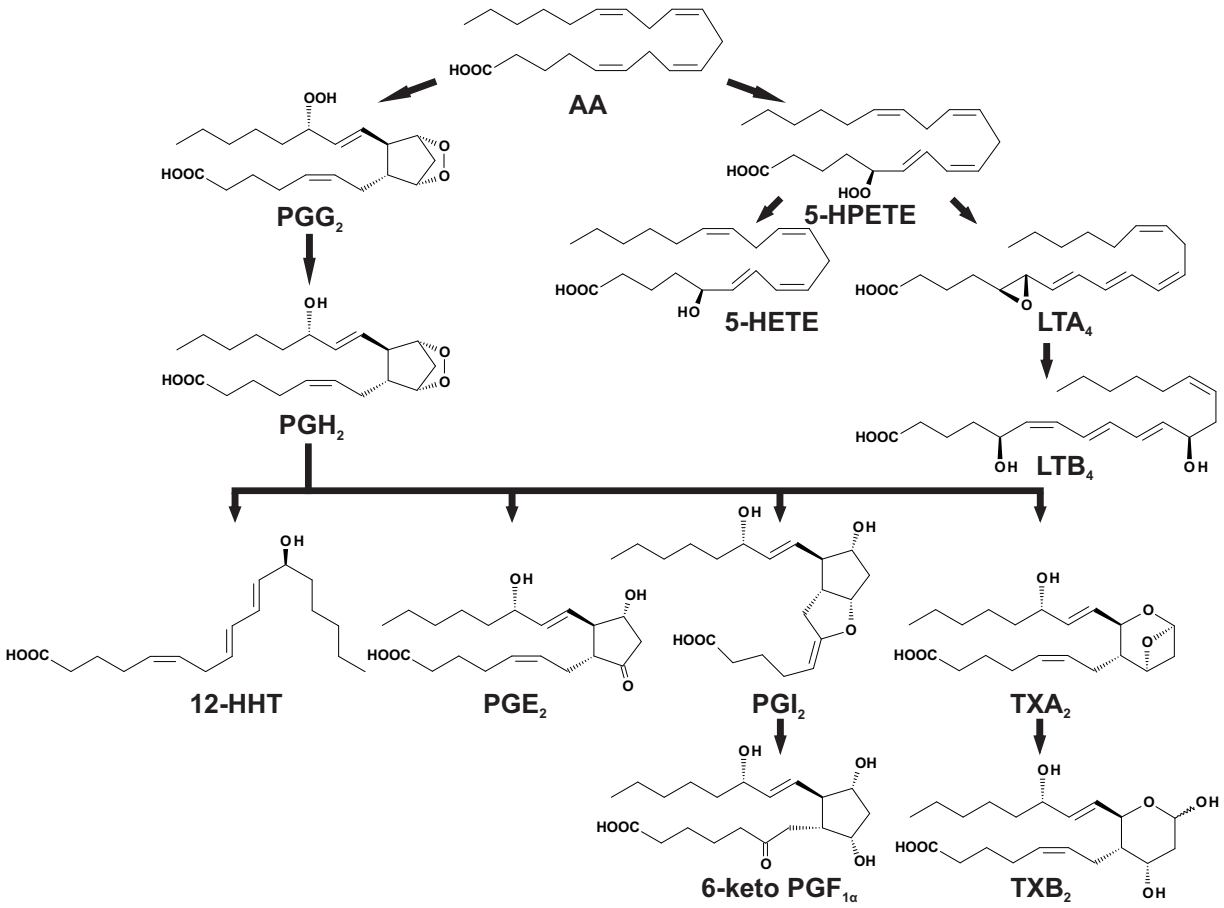


Figure 2: Biosynthesis of selected arachidonic acid metabolites. Arachidonic acid (AA), prostaglandin (PG), leukotriene (LT), thromboxane (TX), 12(S)-hydroxy-5-cis-8,10-trans-heptadecatrienoic acid (12-HHT), 5(S)-hydro(pero)xy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-H(P)ETE).

2.2.2 Phospholipases

2.2.2.1 Classification

Phospholipases catalyze the cleavage of phospholipids from cellular membranes, with the family of phospholipase A_2 (PLA_2s) preferentially cleaving the acyl-moieties in the *sn*-2 position. This way, PLA_2s release unsaturated fatty acids, where lysophospholipids remain, both of which can be metabolized into highly bioactive mediators [60]. Special lysophospholipids (lysoPAF) are acetylated by specific acetyltransferases, leading to the pro-inflammatory platelet activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-3-phosphocholine), a lipid that among other actions, induces platelet aggregation and anaphylaxis [61]. AA is one of the released fatty acids and represents the precursor of LTs, PGs and TXs (Figure **1**).

Up to now, fifteen groups of PLA_2s, comprising different subgroups were characerized [5]. The numbering of these groups arose from the historical discovery, whereas the classification in five categories sheds light on their regulation and function (Table **1**).

The secreted PLA_2s ($sPLA_2$s) represent the most diverse category, including the groups I, II, III, V, IX, X, XI, XII, XIII and XIV. They comprise various venoms from snakes, scorpions and bees, but also human PLA_2s such as the pancreatic PLA_2s (group IB and IID), PLA_2s in macrophages (group V) and leukocytes (group X) and a PLA_2 found in platelets and in synovial fluid from arthritic patients (group IIA) [60]. The $sPLA_2$s are characterized by a low molecular weight and a Ca^{2+}-dependent catalytic dyad composed of histidine and aspartic acid [62]. As indicated by the name, $sPLA_2$s commonly act extracellularly. Group IIA and V PLs have been associated with inflammatory diseases [63-64], but they rather operate coordinated with cytosolic $PLA_2\alpha$ ($cPLA_2\alpha$) activity than directly leading to AA release [5, 65-71]. Extracellularly, $sPLA_2$s exhibit potent anti-bacterial properties [72-73]. An $sPLA_2$ (group IIA) inhibitor was evaluated in clinical trials in the treatment of rheumatoid arthritis, but did not exhibit anti-inflammatory or anti-rheumatic effects [74]. In contrast to group II $sPLA_2$, group X $sPLA_2$ features high affinity to mammal extracellular membranes [75-76], which accounts for its special role in AA supply for CysLT synthesis in bronchial asthma [77]. Part of the effects elicited by $sPLA_2$s may be based on the specific binding to membranous receptors [68, 78].

The Ca^{2+}-independent PLA_2s ($iPLA_2$, group VI) not only cleave phospholipids but also show transacylase activity [79]. Presumably, their main function is the maintenance of membrane homoeostasis. Experiments in $iPLA_2$ knockout mice revealed changes in bone formation [80], apoptosis [81], insulin secretion [82], sperm development [83] and susceptibility to obesity [84]. Furthermore, $iPLA_2\beta$ plays a role in chemotactic migration of monocytes [85].

The category of platelet activating factor acetylhydrolases/lipoprotein-associated phospholipases A_2 (PAF-AH/$LpPLA_2$) comprises enzymes that cleave acyl-moieties that are up to nine carbons long [86]. They cleave and inactivate PAF but also degrade lipids that comprise oxidized short acyl-moieties, which arose from oxidative stress [87]. PAF-AHs display anti-inflammatory actions in acute inflammation [88]. On the other hand, they are established biomarkers of coronary heart diseases [89-90]. A selective inhibitor of $LpPLA_2$, darapladib (SB-480848), is currently subject of a phase III clinical trial (estimated completion date 10/2012 [91]) and is supposed to reduce cardiovascular risk arising from unstable atherosclerotic lesions.

The most recently identified group of phospholipases (group XV) comprises one single enzyme, the lysosomal PLA_2 ($LPLA_2$). $LPLA_2$ was found in alveolar macrophages, exhibits transacylase activity, synthesizes 1-*O*-acylceramides [92] and is associated with the catabolism of pulmonary surfactant [93-94].

The category of cytosolic PLA_2s contains six members ($cPLA_2\alpha$ – ζ). They are composed of a Ca^{2+}-dependent lipid binding C2 domain and a catalytic hydrolase domain, except for the

constitutively membrane bound cPLA$_2$γ [95], which lacks the C2 domain and the associated Ca^{2+}-dependency [96]. The subtype cPLA$_2$α has been examined very extensively as it is expressed in a broad variety of cells and represents the only PLA$_2$ that preferentially cleaves substrate containing AA in the *sn*-2 position [97-98]. It also possesses minor lysophospholipase and transacylase activity. Its regulation will be discussed in the following chapters. Enzyme activity of cPLA$_2$β is inferior to cPLA$_2$α [96, 99] and it is less selective for cleavage at the *sn*-2 position. cPLA$_2$δ was originally discovered in the upper epidermis of psoriatic lesions, where it is supposed to play an essential role in lipid signalling [100].

Table 1: Classification of phospholipases based on [60, 92, 101-103].

group		common sources	size [kDa]	catalytic mechanism	functional classification
I	A	cobra, krait venom	13-15	His-Asp	sPLA$_2$
	B	mammal pancreas	13-15	His-Asp	sPLA$_2$
II	A	human synovial fluid, thrombocytes, rattlesnake, viper venom	13-15	His-Asp	sPLA$_2$
	B	gaboon viper venom	13-15	His-Asp	sPLA$_2$
	C	rat, mouse testis	15	His-Asp	sPLA$_2$
	D	human, mouse pancreas / spleen	14-15	His-Asp	sPLA$_2$
	E	human, mouse brain / heart / uterus	14-15	His-Asp	sPLA$_2$
	F	human, mouse testis skin	16-17	His-Asp	sPLA$_2$
III		bee, lizard, scorpion, human	15-18	His-Asp	sPLA$_2$
IV	A	human U937 cells, thrombocytes, monocytes, neutrophils; RAW 264.7, rat kidney	85	Ser-Asp	cPLA$_2$α
	B	human pancreas / liver / heart / brain	114	Ser-Asp	cPLA$_2$β
	C	human heart / skeletal muscle	61	Ser-Asp	cPLA$_2$γ
	D	mouse placenta, human psoriatic skin	~100	Ser-Asp	cPLA$_2$δ
	E	mouse thyroid / heart / skeletal muscle	~100	Ser-Asp	cPLA$_2$ε
	F	mouse thyroid	~100	Ser-Asp	cPLA$_2$ζ
V		mammal heart / lung / macrophage	14	His-Asp	sPLA$_2$
VI	A-1	P388D$_1$ macrophages, CHO	84-85	Ser-Asp	iPLA$_2$α
	A-2	human B lymphocytes / testis	88-90	Ser-Asp	iPLA$_2$β
	B	human heart / skeletal muscle	88	Ser-Asp	iPLA$_2$γ
VII	A	human, mouse, porcine, bovine plasma	45	Ser-His-Asp	PAF-AH
	B	human, bovine liver / kidney	40	Ser-His-Asp	PAF-AH (II)
VIII	A	human brain	26	Ser-His-Asp	PAF-AH Ib α$_1$
	B	human brain	26	Ser-His-Asp	PAF-AH Ib α$_2$
IX		snail venom (conodipine M)	14	His-Asp	sPLA$_2$
X		human spleen / thymus / leukocyte	14	His-Asp	sPLA$_2$
XI	A	green rice shoots	12.4	His-Asp	sPLA$_2$
	B	green rice shoots	12.9	His-Asp	sPLA$_2$
XII		mammal heart / kidney / skin / muscle	18.7	His-Asp	sPLA$_2$
XIII		parvovirus	< 10	His-Asp	sPLA$_2$
XIV		symbiotic fungus / *Streptomyces*	13-19	His-Asp	sPLA$_2$
XV		alveolar macrophages	45	Ser-His-Asp	LPLA$_2$

2.2.2.2 Pathophysiological role of cytosolic phospholipase $A_2\alpha$

$cPLA_2\alpha$ is supposed to be the main phospholipase accounting for AA release in inflammation [95], though further enzymes subordinately contribute to AA supply [68]. The participation of individual PLA_2s in AA release is dependent on the status of the inflammatory progress [104] and the stimulus [105]. Interaction and mutual regulation of PLA_2s even complicate the assessment of their individual contribution to overall PLA_2 activity [65-67, 69-71]. However, a major role of the $cPLA_2\alpha$ in inflammatory AA release is indisputable [95] and thus this enzyme represents a potential target for anti-inflammatory therapy (see chapter 2.2.2.5).

$cPLA_2\alpha$ knockout mice develop normally, apart from diminished fertility of female mice [106] and a defect of the renal concentration system [107]. Isolated macrophages of LPS-, calcium ionophore A23187- or casein-challenged $cPLA_2\alpha$-deficient animals are unable to produce PGE_2, LTB_4, CysLTs or PAF and the animals are resistant to collagen-induced arthritis [108] and less prone to artery occlusion-induced cerebral infarction, oedema formation and cerebral ischemia [109]. Ovalbumin-induced anaphylactic responses and methacholine-induced bronchial reactivity are significantly decreased [106], due to reduced reactivity of mast cells [110]. Bleomycin-induced pulmonary fibrosis [111], high-fat diet-induced fatty liver disease [112], sepsis- or acid-induced lung injury (adult respiratory distress syndrome) [113], small intestinal polyposis [114], PGE_2-mediated bone resorption [115] and experimental autoimmune encephalomyelitis (animal multiple sclerosis model) [116] are less severe or totally absent in $cPLA_2\alpha^{(-/-)}$ mice. Collagen-induced TXB_2 formation in $cPLA_2\alpha^{(-/-)}$ platelets is extensively reduced, accounting for reduced tendency to thromboembolism and increased bleeding times [117]. In an experimental sepsis model, peritoneal levels of $PGF_{2\alpha}$, PGD_2, LTB_4 and at later time points also IL-6 are diminished [118]. In contrast, colon tumourigenesis is increased in $cPLA_2\alpha$-deficient mice, an effect that is supposed to be due to reduced ceramide-mediated apoptosis [119].

A case report of a human patient with inherited $cPLA_2\alpha$ deficiency revealed similar effects in humans [120]. The production of TXB_2, 12-HETE and LTB_4 in A23187-stimulated human whole blood were markedly reduced. Adenosine diphosphate- and collagen-induced platelet aggregation was diminished, whereas AA-induced platelet aggregation was not altered. Metabolite levels of PGE_2, PGI_2, PGD_2 and TXA_2 in the urine were reduced. Furthermore, the patient suffered from multiple small intestinal ulcers.

In substance P- and carrageenan-induced hyperalgesia in rats, treatment with a common inhibitor of $cPLA_2\alpha$ and $iPLA_2\alpha$ blocks hyperalgesic reactions, which was associated with reduced intrathecal PGE_2 levels [121]. The reduction of eicosanoid formation in whole blood experiments

and of PG synthesis in air pouch and carrageenan-induced paw oedema models after treatment with cPLA$_2$α inhibitors (efipladib and WAY-196025) clearly illustrates the anti-inflammatory potential of these drug candidates [122] (see chapter 2.2.2.5).

2.2.2.3 Structure and catalysis of the cytosolic phospholipase A$_2$α

cPLA$_2$α is an 85 kDa-sized protein (749 amino acids) composed of an N-terminal Ca^{2+}-binding C2 domain (138 amino acids) and a C-terminal catalytic domain linked by a short and flexible tether (amino acids 139-143, Figure **3 A**) [123-125].

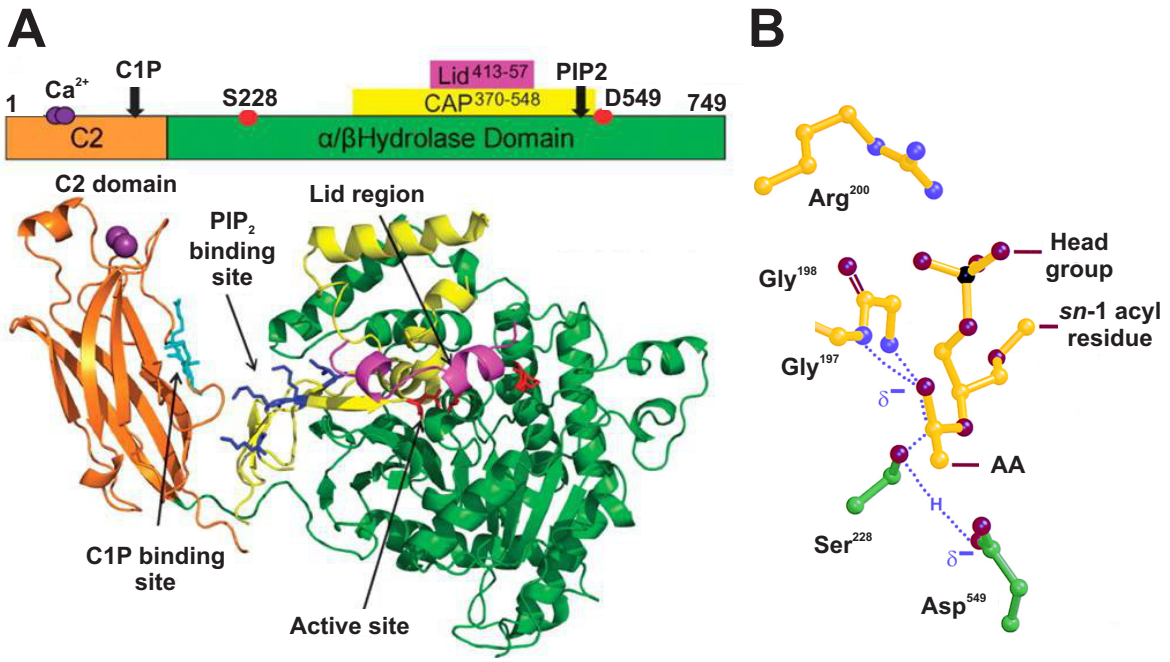


Figure 3: Crystal structure of cPLA2α (A) and the assumed transition-state complex (B). [5, 125].

The C2 domain is responsible for the Ca^{2+}-dependent targeting and binding of distinct membranes [126] (see chapter 2.2.2.4). It is composed of eight β-strands that are connected by three Ca^{2+}-binding loops, which are able to coordinate two Ca^{2+} ions [127]. Ca^{2+} chelation enables lipophilic amino acid residues in two of these loops to penetrate the membrane [128-129] and allows polar amino acid residues of the Ca^{2+}-binding loops to interact with the phospholipid head groups of the membrane [127-128, 130-131]. Furthermore, the C2 domain includes a binding site (cationic β-groove) for the sphingolipid ceramide-1-phosphate (C1P) (see chapter 2.2.2.4) [132]. The C2 domain does not contribute to the enzyme's specificity for AA [126].

The enzymatic activity of the catalytic domain itself is independent of Ca^{2+} [123]. A unique feature of the catalytic domain is a cap/lid region, which prevents the access of substrate to the active site

until activation [133-134]. A conformational change of the enzyme opens the lid, which is assumed to be induced by electrostatic repulsion of anionic residues of the lid on the one side and the phosphate- and anionic phospholipid-borne anionic membrane surface on the other side [135]. Upon opening, a lipophilic region is exposed and stabilized by interaction with the membrane, giving rise to the so-called interfacial activation of the enzyme.

Some amino acid residues on the membrane-oriented surface of the catalytic domain are also implicated in membrane binding by interaction with lipids or proteins [136]. One cluster of basic residues was identified as binding site for polyphosphoinositides (PIPs) [137] (see chapter 2.2.2.4). As mentioned before, the cPLA$_2$α specifically cleaves AA from phospholipids, which is attributed to the special architecture of the hydrophobic active site funnel system [125]. The spatial dimensions of this funnel allow the specific entering of AA due to its characteristic angulation that arises from the *cis* double bond in position C-5.

The catalytic centre of the cPLA$_2$α contains a catalytic dyad composed of Asp^{549} and Ser^{228} (Figure **3 B**) [138-139]. Arg^{200} was found to be essential for the enzyme's activity [138], which is presumably due to a contribution to the binding of the substrate's phosphate-moiety or to the stabilization the oxyanion transition state of the arachidonic acyl-moiety [125].

2.2.2.4 Regulation of the cytosolic phospholipase A$_2$α

As delineated below, cPLA$_2$α is regulated on the transcriptional and post-transcriptional level. Principally, the post-transcriptional mechanisms may affect the affinity of the enzyme to membranes, which is prevalently driven by the C2 domain [128], or they directly change the enzymatic activity of the catalytic domain [140].

Transcriptional regulation

The gene encoding human cPLA$_2$α is located on chromosome 1q25, next to the gene encoding COX-2 [141]. But unlike COX-2, cPLA$_2$α is constitutively expressed in most cells and tissues [142-144]. Transcription is induced after stimulation with pro-inflammatory cytokines, such as IL-1α [145], tumour necrosis factor α (TNFα) [146], macrophage colony stimulating factor (M-CSF) [147] and LPS [148]. Glucocorticoids repress the cytokine-induced upregulation of cPLA$_2$α [145-146, 149]. The promoter of the cPLA$_2$α gene contains several interferon-γ (IFNγ) response elements and two glucocorticoid response elements [150]. cPLA$_2$α expression is induced by platelet-derived growth factor (PDGF) or thrombin through signal transducer and activator of transcription 3 (STAT3) [151-152] and by phorbol myristate acetate (PMA) through c-Jun/Sp1 and c-Jun/nucleolin complexes [153]. A co-repressor for homoeodomain transcription factors, the homoeodomain-interacting protein kinase-2 (HIPK2), was found to restrain cPLA$_2$α transcription

[154]; increased HIPK2 levels might account for the generation of familial adenomatous polyposis, which results in colorectal cancer. Increased expression of cPLA$_2$α was found in several types of cancer and was associated with the tumour-promoting role of PGE_2 [155]. However, the role of cPLA$_2$α in tumourigenesis is controversially disputed, indicating a tissue specific impact of dysregulated cPLA$_2$α [95].

Regulation by membrane binding and cellular trafficking

cPLA$_2$α does not only need phospholipids as substrate *per se*, but also an intact phospholipid membrane for interfacial activation [135]. cPLA$_2$α is typically situated in the cytosol [97]. Upon stimulation with Ca^{2+}-mobilizing agents, two Ca^{2+} ions are bound to the C2 domain, which leads to a conformational tightening of the C2 domain [134] and enhances the affinity to membrane surfaces by neutralizing the anionic charge of the Ca^{2+}-chelating residues that accomplish electrostatic repulsion in the unbound state. Since the truncated C2 domain targets the same membranous structures as the entire cPLA$_2$α protein, the catalytic domain seems not to be essential in Ca^{2+}-dependent translocation [128]. Upon stimulation with Ca^{2+}, cPLA$_2$α usually translocates to the nuclear envelope, endoplasmatic reticulum and Golgi [156-160]. Low (100-125 nM) or transiently elevated Ca^{2+} levels provoke preferential translocation to the Golgi, whereas additional translocation to the endoplasmatic reticulum and perinuclear membrane occurs in case of sustained Ca^{2+} levels (> 210-280 nM) [156].

The targeting of intracellular membranes is attributed to preferential binding of the Ca^{2+}-triggered lipophilic Ca^{2+}-binding loops to membranes that are rich in zwitterionic phosphatidylcholine, whereas membranes that contain important amounts of (an)ionic lipids (e.g. the plasma membrane) are disfavoured [161-162].

As translocation of cPLA$_2$α in cellular models already occurs at sub-micromolar Ca^{2+} levels but requires higher concentrations in cell-free models [163], additional mechanisms seem to potentiate the binding of natural membranes. Binding of C1P to the C2 domain was identified as supplementary binding principle [132] and increases cPLA$_2$α activity [164]. C1P is produced by ceramide kinase, which is located in the Golgi [165] and siRNA-mediated downregulation of this enzyme blocks cPLA$_2$α translocation, agonist-induced AA release and PG production [166-167]. Thus, targeting of the Golgi may be explained by binding of C1P in combination with the Ca^{2+}-induced raise in the C2 domain's affinity for phosphatidylcholine membranes.

cPLA$_2$α translocation may also occur independently of Ca^{2+}. AA induces the translocation of cPLA$_2$α or its truncated C2 domain to phosphatidylcholine-rich intracellular membranes [168]. The concrete mechanism of this pathway has not been elucidated yet but it is seemingly not mediated by AA metabolites or through the activation of G protein-coupled receptors.

In some cases, cPLA$_2$α also translocates to cellular plasma membranes; e.g. in PMA-stimulated glomerular epithelial cells [169]. Upon stimulation of granulocytes with PMA, zymosan or formyl-methionyl-leucyl-phenylalanine (fMLP), cPLA$_2$α transiently translocates first to the plasma membrane and then to the nuclear membrane [170]. In human platelets, mechanical or thrombin-induced activation leads to cPLA$_2$α translocation to the plasma membrane [171]. This membrane association is resistant to the Ca^{2+}-chelating agent EGTA but is abolished upon de-polymerization of actin filaments.

Regulation by phosphorylation

Three phosphorylation sites have been identified in cPLA$_2$α [172-173]; all of them are located in the catalytic domain. Ser^{505} is phosphorylated by the mitogen-activated protein kinases (MAPK) extracellular signal-regulated kinase-1/-2 (ERK-1/-2) [174], p38 [175] and c-Jun N-terminal kinase (JNK) [176-177], Ser^{515} is phosphorylated by Ca^{2+}/calmodulin-dependent kinase II (CaMKII) [173], and Ser^{727} is phosphorylated by mitogen-activated protein kinase interacting kinase 1 (MNK1), mitogen- and stress-activated protein kinase 1 (MSK1) and p38-regulated/activated protein kinase 1 (PRAK1) [178]. Completely dephosphorylated cPLA$_2$α is catalytically active and phosphorylation at Ser^{505} leads to a 3-fold increase in enzymatic activity [179], whereas phosphorylation of irreversibly membrane-bound cPLA$_2$α only increases AA release about 1.3-fold [180]. In platelets, phosphorylation at position Ser^{505} enhances the catalytic activity without reducing the requirement for Ca^{2+} [181]. However, more detailed experiments applying site-directed mutagenesis indicate that phosphorylation of Ser^{505} critically enhances the membrane attachment at physiological Ca^{2+} levels (in the submicromolar range) but does not affect cPLA$_2$α translocation and activity at high Ca^{2+} levels [140]. DAS et al. suggested a conformational change of cPLA$_2$α upon phosphorylation at Ser^{505}, leading to membrane penetration of hydrophobic residues that are situated next to the active site, which sustains the membrane interaction even after decline of the Ca^{2+} levels.

Ser^{727} is selectively phosphorylated by MNK1, MSK1 and PRAK1. These kinases are activated by ERK-1/-2 and p38 MAPK and therefore, phosphorylation of Ser^{505} is likely to be accompanied by phosphorylation at Ser^{727} [178, 182]. Phosphorylation of Ser^{727} leads to increased enzymatic activity of cPLA$_2$α [183], which in analogy to phosphorylation of Ser^{505}, is more distinctive at low Ca^{2+} levels but seems to be negligible at higher Ca^{2+} concentrations [140]. Recently, TIAN et al. discovered that phosphorylation of Ser^{727} activates cPLA$_2$α by disruption of the inhibitory complex of cPLA$_2$α and A2t, a complex composed of p11 (S-100A10/calpactin I light chain) and annexin II [184].

Regulation of cPLA$_2$α by CaMKII appears to be complex and is not fully understood yet. Phosphorylation of Ser^{515} by CaMKII is associated with increased cPLA$_2$α activity [173]. However, the suppressive effect of calmodulin or CaMKII inhibitors on cPLA$_2$α activity is conserved in a S515A cPLA$_2$α mutant [185], suggesting further regulatory mechanisms of CaMKII that are independent of Ser^{515} phosphorylation. CaMKII-mediated effects on cPLA$_2$α activity are frequently accompanied by activation of MAPK and the associated phosphorylation of Ser^{505} [186-187]; nevertheless these pathways are not stringently connected [188-189].

Interaction with proteins and lipids

Several proteins have been identified interacting with cPLA$_2$α, mostly regulating cPLA$_2$α translocation and membrane binding. Vimentin [190-191], actin [171, 192], cPLA$_2$α-interacting protein (PLIP) [193], cPLA$_2$α-activating protein (PLAP) [194-198] and inducible nitric oxide synthase (iNOS) [199] increase cPLA$_2$α activity in cellular systems. On the other hand, caveolin-1 [200], p11/annexin II complex [184, 201-202], annexin I [203-204], annexin I/p11/cystic fibrosis transmembrane conductance regulator (CFTR) multiprotein complex [205], annexin III and V [202, 206] and annexin VI [207] are negative modulators of cPLA$_2$α activity.

cPLA$_2$α exhibits a high specificity for AA in the *sn*-2 position of phospholipids [98]. Membranes that contain high levels of phosphatidylinositole or phosphatidylserine represent the main reservoir of *sn*-2-bound AA [208]. However, membrane binding is primarily carried out by the C2 domain, and thus membranes that are rich in phosphatidylcholine are preferentially targeted and provide the main share of AA [209]. Anionic lipids activate the enzymatic activity, an effect that is attributed to the electrostatic repulsion of the lid region during interfacial activation of the enzyme [135]. Anionic lipids in the membrane are also implicated in the binding of cationic patches of the catalytic domain, which enhances AA release especially at low Ca^{2+} levels [210]. On the other hand, anionic lipids that exceed a critical amount lead to repulsion of the lipophilic C2 domain [211].

Some membranous lipids provoke enhanced cPLA$_2$α activity by facilitating membrane accessibility. Ceramides and diacylglycerols disturb the lamellar structure of membranes to inverse hexagonal structures [212-213] and thereby may facilitate the access of the enzyme to its substrate [214-215]. Several studies demonstrated the stimulatory effect of ceramides and diacylglycerols in cell-free [211, 216] and various cellular models [216-221]. In addition, membrane-incorporated cholesterol was reported to stimulate cPLA$_2$α activity in a cellular and non-cellular context [216, 222].

The C2 domain of cPLA$_2$α exhibits a binding site for C1P [132], which mediates preferential targeting of distinct membranes [167] (see chapter 2.2.2.3 and 2nd paragraph of this chapter); C1P

binding leads to enhanced cPLA$_2$α activity and reduces the threshold level for Ca^{2+}-induced activation [164, 166].

Furthermore, the catalytic domain of cPLA$_2$α contains a binding site for polyphosphoinositides (PIPs) [137] (see chapter 2.2.2.3). Phosphatidylinositol-4,5-bisphosphate (PIP$_2$) is mainly integrated in the inner leaflet of plasma membranes and lower levels are found on the endoplasmatic reticulum, the nuclear envelope and the Golgi [223]. Binding of PIP$_2$ decreases the Ca^{2+} concentration required for full activation of cPLA$_2$α [211] and stimulates cPLA$_2$α activity, distinctively exceeding the effect of other anionic lipids [224]. DAS et al. proposed a PIP$_2$-induced conformational change of the C2 domain against the catalytic domain that optimally positions the catalytic centre in respect of its substrate [135]. Even in the absence of Ca^{2+}, PIP$_2$ enables cPLA$_2$α to bind phosphatidylcholine membranes and to release AA [137]. In cellular models, stimulation or inhibition of PIP$_2$ strikingly modulates cPLA$_2$α activity [225-226].

2.2.2.5 Inhibitors of the cytosolic phospholipase A$_2$α

AA-analogues like methoxy arachidonyl fluorophosphonate (MAFP) and the trifluoromethyl ketone AACOCF$_3$ represent the first generation of cPLA$_2$α inhibitors and irreversibly bind the Ser^{228}-residue of the catalytic dyad [227]. These compounds possess IC$_{50}$ values in the micromolar range in cellular models and are only little effective on sPLA$_2$α (group IIA) [228]. However, they unselectively affect AA-metabolism in polymorphonuclear leukocytes (PMNL) [229] and feature high cytotoxicity that is most likely based on their amphiphilic structure [230].

Several choline derivatives inhibit cPLA$_2$α, acting by incorporation in the membrane and competing with phosphatidylcholine for binding of cPLA$_2$α binding. These compounds exhibit IC$_{50}$ values in the low micromolar range, have about 70-fold specificity for cPLA$_2$α compared to sPLA$_2$ (group IIA) and do not interfere with PLC, PLA$_1$ or PLD [231].

2-Oxoamide derivatives inhibit cPLA$_2$α in cell-free, cellular and *in vivo* models [232] by binding in proximity to the active site [233]. Selected derivatives suppress PGE$_2$ production in stimulated macrophages with IC$_{50}$ values of 5 µM [232] and inhibit carrageenan-induced paw oedema in rats with ED$_{50}$ values of 0.02 mg/kg [234]. Analgesic [234] and anti-hyperalgesic activity [121] were stated in pain models in rats.

A variety of pyrrolidine (or pyrrophenone) derivatives inhibit cPLA$_2$α with IC$_{50}$ values in the nanomolar range in cellular and cell-free models [235] and in human whole blood [236]. Pyrrolidine 1 exhibits 17-fold selectivity for cPLA$_2$α compared to cPLA$_2$γ, more than 100-fold selectivity compared to iPLA$_2$β and it does not perceivably affect sPLA$_2$ activity (group IIA, V and X) [237]. Automated molecular docking studies and deuterium exchange mass spectrometry

revealed interaction of a pyrrolidine-type inhibitor with the active site Ser^{228} combined with numerous lipophilic binding events distal from the active site [233]. In collagen-induced arthritis in mice, pyrrolidine-type inhibitors markedly reduce the symptoms of arthritis, osseous destruction, the expression levels of $cPLA_2\alpha$, the production of PGE_2 and LTB_4 and the mRNA levels of matrix metalloproteinases (MMP-3, -8, -9, -13) and COX-2 [238]. It should be noted that the required doses for the effects were rather high (30 to 100 mg/kg twice per day).

Another lead structure of modern $cPLA_2\alpha$ inhibitors comprises an indole and a benzyl sulphonamide moiety, yielding the most common derivatives efipladib and WAY-196025, with submicromolar IC_{50} values in cell-free and human whole blood models [239-240]. They also display oral efficacy in rat carrageenan-induced air pouch and paw oedema models as well as in carbachol-induced bronchoconstriction in sheep. Nevertheless, these compounds offer poor bioavailability of maximally 16% after oral administration in dogs, which may be explained by their high molecular weight and lipophilicity [240].

LEHR and co-workers developed different inhibitors based on an indolylpropanone scaffold [241] that inhibit cell-free or cellular $cPLA_2\alpha$ activity in submicromolar concentrations [241-242]. However, they are extensively metabolized by liver microsomes [243] and offer poor availability upon peroral application in mice [244]. Intravenously applied drug is rapidly cleared from the plasma but the inflammatory reaction in a murine model of contact dermatitis was significantly suppressed after topical administration [244].

Only few natural compounds have been described as inhibitors of $cPLA_2\alpha$ activity so far. Variabilin, a sesterterpene isolated from the marine sponge *Ircinia variabilis* was found to inhibit $sPLA_2$ (group IIA) and $cPLA_2\alpha$ in cell-free and cellular assays with IC_{50} values of 7 and 8 µM, respectively [245]. PMA-induced but not AA-induced mouse ear oedema was suppressed by topical administration of variabilin and peroral administration inhibited PGE_2 and LTB_4 formation in a murine air pouch model. Recently, lutein was found to inhibit $cPLA_2\alpha$ with an IC_{50} value of 14 µM and without affecting $sPLA_2$ activity [246]. Abruquinone A, an isoflavanquinone from *Abrus precatorius* inhibits AA release and subsequent TXB_2 and LTB_4 production from A23187- and fMLP-stimulated rat neutrophils [247]. This inhibition is not due to direct inhibition of the catalytic domain, but is based on inhibition of ERK and the blockade of Ca^{2+} mobilization.

2.2.3 Cyclooxygenases

2.2.3.1 Classification

Cyclooxygenases (COX) are haeme-dependent bis-oxygenases with peroxidase activity [248]. Although research on PGs and COX emerged in the 1930s, the existence of two distinct enzymes

was only identified in the early 90s [249]. The two isoenzymes share ~60% homology in their amino acid sequence, catalyze the same reactions by the same mechanism, exhibit similar conformation of the catalytic and substrate-binding regions and have a similar molecular mass of 70 kDa [7]. They markedly differ in their transcriptional regulation, tissue distribution and the dimension of the COX substrate channel.

2.2.3.2 Structure and catalysis of cyclooxygenases

COX-1 and -2 are membrane-bound proteins composed of approximately 600 amino acids and reside as homodimers primarily on lumenal membranes of the endoplasmatic reticulum and inner and outer membranes of the nuclear envelope [250-252]. COX proteins contain four distinct domains; an N-terminal signalling peptide that is cleaved after synthesis and trafficking of the enzyme and three more domains responsible for dimerization, membrane binding and catalysis [248].

The signalling peptides of COX-1 and COX-2 differ in length and lipophilicity, which was associated with differential targeting of the proteins after synthesis [10]. Dimerization only takes place within one isoform and occurs by non-covalent interactions of the dimerization domains of the monomers [10]. The membrane-binding domain is composed of four amphipathic helices with protruding aromatic residues that interact with one leaflet of the membrane bilayer [253]. The helices form an opening that represents the putative access for AA or inhibitors to the cyclooxygenase active site. The largest part of the enzyme is the C-terminal catalytic domain, which contains two distinct active sites for the peroxidase and the cyclooxygenase reaction [253].

The cyclooxygenase active site is a hydrophobic channel with an opening faced to the membrane. Arg^{120} (all numbering refers to COX-1) in the channel is responsible for the binding of the substrate's carboxylic group [254]. The catalytic pocket in the very end of the channel contains the Tyr^{385}-residue that attacks AA as a tyrosyl radical, abstracts hydrogen thereby forming an arachidonyl radical, which undergoes cyclization and oxygenation to PGG_2 [255-257]. The active site channel of the respective COX isoforms only differs by two amino acid residues (Ile^{523} and Ile^{434} in COX-1 and valine residues in COX-2), leading to a wider channel in COX-2, which was exploited for the development of COX-2-selective inhibitors [258]. Ser^{530} in the catalytic pocket plays an important role in the deactivation of COX by acetylsalicylic acid as it is irreversibly acetylated by the drug.

The peroxidase site is situated remote from the membrane in a shallow cleft that also contains the binding site for haeme, which is fixed by His^{388} [253]. Peroxidase and cyclooxygenase activity are interrelated by the initial generation of the cyclooxygenase's tyrosyl radical through the

peroxidase's ferryl-oxo porphyrin radical intermediate [259-260]. This central step of the so-called branched chain model suggests the activation of the haeme by an endogenous oxidant (e.g. a hydroperoxide), which primarily activates the cyclooxygenase's tyrosine residue. Thereby the cyclooxygenase cycle is activated and perpetuates the regeneration of its tyrosyl radical by itself.
In the presence of sufficient substrate, COX enzymes undergo suicide inactivation within 1-2 min [10], presumably by self-destructive peroxidase side reactions of the ferryl-oxo haeme [261].
The kinetic properties, the turnover numbers, and the susceptibility to suicide inactivation of the COX isoforms are widely comparable [262-265]. However, in cells in which both isoforms are expressed, AA is preferentially converted by COX-2 [266]. This is most likely due to negative allosteric regulation that occurs at submicromolar AA concentrations for COX-1 but not for COX-2 [266-268]. The negative allosteric regulation is abolished in the presence of excess of hydroperoxide [269]. Regarding the kinetics of the peroxidase reaction, COX-2 was found to require only 10% of the hydroperoxide concentration that was needed for COX-1 activation [270].

2.2.3.3 Regulation of cyclooxygenases

Transcriptional and translational regulation

Transcription represents a central checkpoint in COX regulation and differentiates the roles of the respective isoenzymes. COX-1 is constitutively expressed in most tissues, prominently in endothelia, the renal collecting tubules, seminal vesicles, monocytes and platelets [248]. COX-1 expression in these cells is induced during cell differentiation e.g. via Sp1 elements [271]. However, COX-1 appears to be inducible in the course of LPS-induced inflammation [272], though the underlying mechanism remains to be elucidated.
In contrast, various pro-inflammatory cytokines, mediators and pathways control the transcription of COX-2 [248]. The COX-2 gene is responsive to IL-1α/β [273-274], TNFα [274], LPS [274-276], PMA [249] and diverse growth factors and cytokines [248] whereas its transcription is suppressed by glucocorticoids [276] and anti-inflammatory cytokines (IL-4/-10) [277-278]. Nuclear factor κ B (NF-κB) [273], CCAAT enhancer binding protein (C/EBP) [279] as well as the MAPK p38 [280], ERK-1/-2 [275, 280] and JNK [281] are involved in the signal transduction. The promoter of the COX-2 gene contains regulatory elements for NF-κB, Sp1, C/EBP (nuclear-factor for IL-6, NF-IL6) and an activating transcription factor/cyclic adenosine monophosphate-responsive element (ATF/CRE) E-box [248]. Furthermore, COX-2 translation is regulated by mechanisms that control COX-2 mRNA stability [282-285]. Nevertheless, constitutive or inducible COX-2 expression contributes to homoeostasis of some cells or tissues without a

pathogenic background [248]. COX-2 is involved in reproduction [286], immunity [287], renal physiology [288], neurotransmission [289], bone resorption [290] and pancreatic secretion [291].

Post-translational regulation and inhibition of cyclooxygenases

The redox tone or the presence of oxidants regulates COX activity, since COX has to be activated for the initiation of the catalytic cycle [259-260] (see chapter 2.2.3.2). Hydroperoxides (e.g. PGG_2, HPETEs) but also peroxynitrite that is formed by coupling of superoxide and NO, account for haeme activation [292-294].

COX activity depends on AA supply and inhibition of PG synthesis is clinically achieved by NSAIDs, which compete with AA for binding at the cyclooxygenase's active site [253]. NSAIDs offer anti-inflammatory, analgesic and anti-pyretic properties [295]. The main drawback of unspecific COX inhibitors is their gastrointestinal toxicity [296]. This side effect may be explained by inhibition of COX-1-derived gastroprotective PGE_2 [297] and was attenuated by the development of inhibitors that are highly selective for COX-2, the so-called coxibs [296]. Unfortunately, long-term therapy with these compounds is accompanied by increased cardiovascular risk that presumably results from reduced production of vascular epithelial PGI_2 [16].

2.2.4 Prostaglandin E_2 synthases

2.2.4.1 Classification of prostaglandin E_2 synthases and their role in inflammatory diseases

To date, three isoforms of prostaglandin E_2 synthases (PGES) have been identified. mPGES-1 was the first isoform that was purified [298], cloned and characterized [299]. mPGES-1 is both, a constitutive and inducible protein and mPGES-1 mRNA was found in human placenta [299], prostate, testis, mammary gland and seminal vesicles [300] as well as in murine urogenital organs [301-302]. mPGES-1 protein is expressed in the lung, spleen, kidney and stomach of mice [303] and constitutive transcription of mPGES-1 mRNA was also reported in human PMNL [304] and rat Kupffer cells [305]. In contrast, in human monocytes and macrophages [304, 306] and rat heart, lung, colon and brain, mPGES-1 mRNA was not detected until stimulation with LPS [307]. Also in human heart and liver, the enzyme was only detected after infarction and hepatitis [308]. Co-transfection experiments demonstrated preferential coupling of mPGES-1 with COX-2 [309].

The second known isoform is the cytosolic PGES (cPGES, p23). This glutathione-dependent enzyme is constitutively expressed in several rat tissues (heart, thymus, liver, spleen, stomach, testis) with exception of the brain where it is induced after LPS challenge [310]. Moreover, cPGES

was isolated and cloned from human brain [311]. Co-transfection experiments of cPGES revealed preferential functional coupling with COX-1 [310]. The specific activity of cPGES is about 100-fold lower than that of mPGES-1 [312].

Another membrane-bound PGES isoform (mPGES-2) was firstly isolated from bovine heart [313]. mPGES-2 mRNA was detected in human brain, heart, lymph nodes, skeletal muscle, kidney, trachea, foetal thymus and lung. Treatment of mice with LPS resulted in modest increase of mPGES-2 expression in liver and colon tissue whereas expression in brain, heart and lung remained on the constitutive level [308]. Exceptionally, mPGES-2 is active in the absence of glutathione, but thiol-reducing reagents increase its activity [314]. The specific activity of recombinant mPGES-2 (cloned from monkey DNA) is comparable to that of cPGES but markedly lower than that of mPGES-1 [312]. mPGES-2 equally metabolizes PGH_2 provided by COX-1 and -2 [308]. mPGES-2 appears not necessarily to be membrane-bound as spontaneous cleavage of the N-terminal anchor sequence releases an active cytosolic protein [309].

mPGES-1 is involved in many inflammatory diseases. Upregulation of mPGES-1 was detected in synovial tissue of patients suffering from rheumatoid arthritis [308, 315-316], in the cartilage of osteoarthritic patients [317] and in the intestinal mucosa in inflammatory bowel diseases [318]. Treatment of rats with IL-1β or LPS leads to expression of mPGES-1 in central nervous structures, which is associated with the development of fever and the processing of pain [13, 319-320]. Data from knockout mice suggest beneficial effects of mPGES-1 blockade in models on arthritis [321-322], fever [13, 323], pain [321-322, 324], atherosclerosis [325] and stroke [326].

In summary, the prominent specific activity of mPGES-1, its regulation by inflammatory mediators (see chapter 2.2.4.3), the mechanistic coupling to other pro-inflammatory enzymes and its involvement in diverse diseases reveals this PGES isoform as the major contributor in pathogenic PGE_2 synthesis. Therefore, mPGES-1 was principally considered in the quest for novel anti-inflammatory enzyme inhibitors. However, experiments using siRNA-induced knock-down of mPGES-1 suggest that its activity may be compensated by alternative PGE_2 synthases in some cells or tissues [327].

2.2.4.2 Structure and catalysis of microsomal prostaglandin E_2 synthase-1

mPGES-1 is a member of the membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) superfamily, which also comprises the LTC_4 synthase and 5-LO activating protein (FLAP) [328]. It is a 17 kDa-sized protein (152 amino acids) that is extremely unstable in the absence of glutathione (half life ~30 min) [298]. Recently, the crystal structure of mPGES-1 in its complex with glutathione was determined [329] (Figure **4 A**). The crystallographic structure is

typical for MAPEG proteins: mPGES-1 is composed of four lipophilic transmembrane α-helices while one molecule of glutathione is coordinated in the cleft of an mPGES-1 homotrimer. These complexes are integrated in the membranes of the nuclear envelope and endoplasmatic reticulum with a central funnel-shaped opening facing the cytosolic side. Glutathione is fixed in this funnel by salt bridges of its carboxylate groups with arginine residues. The substrate is supposed to enter the funnel through a mobile cleft from the interior of the lipophilic membrane [329]. A proposed catalytic mechanism includes the attack of the PGH_2 endoperoxide bridge by the glutathione thiolate (Figure **4 B**) with Arg^{126} serving as proton donator/acceptor.

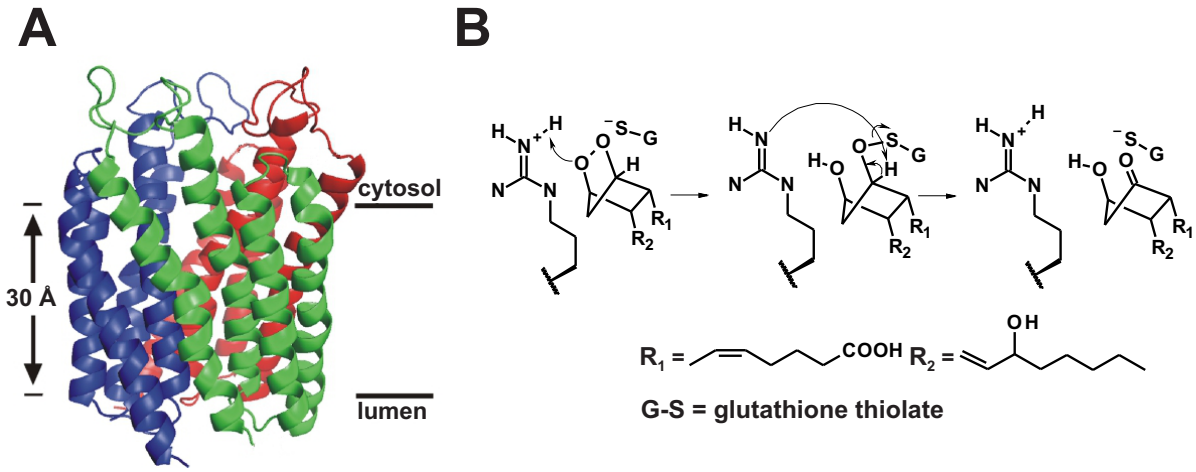


Figure 4: Structure (A) and putative molecular mechanism (B) of mPGES-1. [329] modified.

2.2.4.3 Regulation of microsomal prostaglandin E_2 synthase-1

mPGES-1 is decisively regulated on the transcriptional level (see below). Nevertheless, mPGES-1 activity is post-transcriptionally regulated by direct inhibition through different lipid mediators, e.g. AA and 15-deoxy-Δ(12,14)-PGJ_2 (IC_{50} = 0.3 µM) [330].

Although the mPGES-1 gene is co-regulated with COX-2 [331-333], their promoter regions are quite different. The mPGES-1 promoter contains response elements for the glucocorticoid receptor, AP-1 and two GC boxes [334-335]. Basal transcription of mPGES-1 is mediated by binding of Sp1 and Sp3 transcription factors to the GC boxes [336]. Dependent on the cell type, IL-1β [332, 337], TNFα [318, 331], LPS [338-339] and epidermal growth factor (EGF) [315] induce mPGES-1 via pathways involving JNK [331, 337], ERK and p38 MAPK [332] and the transcription factors early growth response gene 1 (Egr-1) [318, 337, 339], NF-IL6 [338] and NF-κB [331, 337, 339].

On the contrary, both IL-1β- and TNFα-induced mPGES-1 expression are suppressed by dexamethasone [333]. PPARγ activation inhibits IL-1β-induced mPGES-1 expression [340]. The

direct inhibitor of mPGES-1, 15-deoxy-Δ(12,14)-PGJ_2 (see above) was also shown to be a PPARγ-agonist leading to suppressed transcription of mPGES-1 [330, 341], which is potentiated by additional inhibition of the NF-κB pathway [342].
Recently, a mechanism of negative-feedback regulation of mPGES-1 was discovered in LPS-stimulated neuronal tissue. Binding of mPGES-1-derived PGE_2 to EP2 and EP4 receptors blocks LPS-induced TNFα production and mPGES-1 induction *in vitro* and *in vivo* [343].

2.2.5 Lipoxygenases

2.2.5.1 General properties and classification of lipoxygenases

Lipoxygenases (LOs) are non-haeme iron metalloproteins catalyzing the stereoselective insertion of molecular oxygen (dioxygenase) in unsaturated fatty acids [51]. The products are hydroperoxyeicosatetraenoic acids (HPETEs) that are reduced to the respective hydroxyeicosatetraenoic acids (HETEs) or are converted by downstream synthases to LTs or lipoxins [51, 54]. The common nomenclature categorizes human LOs dependent on the site of specific peroxidation (5-, 12- and 15-LO). Six distinct LOs have been identified in humans: 5-LO, 12(S)-LO (platelet type), 12(R)-LO (epidermis type), 12/15(S)-LO (leukocyte/reticulocyte-type), 15(S)-LO (epidermis-type) and the epidermis-type LO 3 [51, 53]. The molecular weight of animal LOs range between 75 and 80 kDa and they are composed of a C-terminal catalytic domain and an N-terminal β-barrel domain that is referred to as C2-like domain [51, 344].

2.2.5.2 Structure and catalysis of 5-lipoxygenase

Human 5-LO is a 78 kDa-sized protein of 674 amino acids in length [345]. Recently, the crystal structure of a stabilized mutant of human 5-LO was elucidated [344]. In analogy to $cPLA_2\alpha$, the C2-like domain mediates Ca^{2+}-dependent binding of the cytosolic 5-LO to cellular membranes [346]. The binding is conferred by tryptophane residues of the putative Ca^{2+}-binding loops that are able to interact with membranes upon binding of two Ca^{2+} ions [347]. Similar to $cPLA_2\alpha$, enzymatic interaction at the water-membrane interface was suggested as a general principle of 5-LO activity [348] and phosphatidylcholine was found to provoke interfacial stimulation [349]. The catalytic domain (~550 amino acids) is characterized by iron that is ligated by three histidine residues and the C-terminal carboxylic group [51].
In the inactive enzyme, the iron exists in the ferrous state (Fe^{2+}) and oxidation to ferric iron (Fe^{3+}) is required for catalytic activity [350]. The oxidized iron subsequently abstracts doubly allylic hydrogen from AA, leading to ferrous iron and a radical that is stabilized by hyperconjugation [351-352]. Oxygen specifically attacks the pentadienyl radical leading to an iron-coordinated

peroxide radical that is converted to the hydroperoxide (5-HPETE) under regeneration of ferric iron. A part of 5-HPETE undergoes dehydration to the highly reactive epoxide LTA_4 by the LTA_4 synthase activity of 5-LO [353]. 5-HPETE, that dissociates from 5-LO before metabolization to LTA_4, is reduced to the corresponding alcohol 5-HETE. Like COX, 5-LO undergoes suicide inactivation within minutes after stimulation [354-355].

2.2.5.3 Regulation of 5-lipoxygenase

Transcriptional regulation

The 5-LO promoter contains several consensus-binding sites for transcription factors like c-myb, activating protein 2 (AP-2), NF-κB, Sp1 [356], Sp3 [357], early growth response genes 1 and 2 (Egr-1/-2) [358], retinoid Z receptor α (RZRα) [359] and retinoic acid receptor-related orphan receptor α (RORα). 5-LO expression prevails in myeloid cells, such as granulocytes, monocytes, macrophages, mast cells and B lymphocytes [360], which is commonly paralleled by FLAP expression. Transforming growth factor β (TGFβ) [361] and calcitriol [362] were found to induce 5-LO expression in leukocyte cell lines during cell maturation. Granulocyte macrophage colony-stimulating factor (GM-CSF) induces 5-LO expression and activity in PMNL [363] and monocytes [364]. Epigenetic regulation by promoter methylation or histone deacetylation is involved in 5-LO gene silencing in non-5-LO-expressing cells [365-366].

Calcium

Calcium (Ca^{2+}) increases the lipophilicity of the C2-like domain and thereby the affinity to the phosphatidylcholine-rich nuclear membrane [346-347]. Thus, Ca^{2+} mediates translocation of 5-LO to the substrate. In cell-free assays using exogenous AA as substrate, Ca^{2+} enhances the enzymatic activity of 5-LO in the presence of membrane structures [349] although Ca^{2+} is not necessary for catalytic activity *per se* [367]. While micromolar concentrations of Ca^{2+} are necessary for increasing 5-LO activity in cell-free models, intracellular Ca^{2+} levels above 150 nM suffice for activation of cellular 5-LO [368]. The threshold Ca^{2+} level for cellular enzyme activation is dependent on additional regulatory mechanisms such as the redox tone [367].

Adenosine triphosphate (ATP)

Nucleotides, especially ATP, stimulate 5-LO activity [369]. The stimulation does not involve ATP cleavage, energy consumption or phosphorylation of 5-LO but is mediated by direct binding to residues in the C2-like and catalytic domain in a stoichiometry of one molecule of ATP per molecule 5-LO [370-371]. Cellular ATP levels in the micromolar range coincide with ATP concentrations that are necessary for activation [372].

Oxidizing agents and lipids

Oxidation of the ferrous iron is a prerequisite for 5-LO activity [350] (see chapter 2.2.5.2). Therefore, lipid peroxides e.g. 5-HPETE, 12-HPETE and 15-HPETE stimulate 5-LO activity in cell-free assays [373-374]. In addition, Fe^{2+} ions that promote lipid peroxidation excite 5-LO activity, whereas reducing agents, such as mercaptoethanol and glutathione inhibit 5-LO product formation [375].

1-Oleoyl-2-acetylglycerol (OAG) stimulates 5-LO activity in the absence of Ca^{2+} via interaction with the C2-like domain [376]; this effect is abolished in the presence of phospholipids and cellular membranes. Besides being the substrate of 5-LO, AA Ca^{2+}-dependently binds to an allosteric site of 5-LO and thereby suppresses its activity [377].

Phosphorylation

Phosphorylation of 5-LO takes place at three serine residues: Ser^{271}, Ser^{663} and Ser^{523}, which are phosphorylated by MAPK-activated protein kinases 2 and 3 (MAPKAPK-2/-3) [378-379], ERK-2 [380] and protein kinase A (PKA) [381], respectively. MAPKAPK-2/-3 are phosphorylated by p38 MAPKs that in turn are activated by cellular stress or inflammatory mediators [382]. Ensuing phosphorylation of 5-LO at Ser^{271} increases its cellular activity [378] and site-directed mutagenesis of Ser^{271} to alanine blocks AA-induced MAPKAPK-2-mediated activation of 5-LO in transfected cells [379]. Cellular stress-induced activation of 5-LO occurs independently of Ca^{2+} [383]. Similarly, the AA-induced ERK-2-mediated phosphorylation of 5-LO at Ser^{663} stimulates its cellular activity, which does not occur in cells expressing mutated 5-LO-S663A [380]. These findings suggest a fundamental role of MAPK-driven phosphorylation events in 5-LO activation by stimuli that do not lead to increased Ca^{2+} levels (e.g. AA). Recently, phosphorylation at Ser^{271} was found to hinder the nuclear export of 5-LO by exportin-1 [384].

On the contrary, PKA-induced phosphorylation of Ser^{523} leads to impaired 5-LO activity in cell-free and cellular models [381, 385]. Furthermore, phosphorylation by PKA provokes the redistribution of 5-LO from the nucleus to the cytoplasm, which can be evoked by increasing levels of intracellular cyclic adenosine monophosphate (cAMP) [385] and can be prevented by unsaturated fatty acids (AA) or competitive 5-LO inhibitors [229].

Thus, AA crucially regulates 5-LO translocation as it inhibits PKA-mediated redistribution of 5-LO to the cytosol and stimulates translocation to the nuclear membrane via MAPK-induced phosphorylation at Ser^{271} and Ser^{663} [386].

Protein interactions

5-LO activating protein (FLAP) is an 18 kDa-sized protein (161 amino acids) with three transmembrane-spanning α-helices and two hydrophilic loops [387-388] acting as a homotrimer [389]. FLAP is localized in the inner and outer nuclear membrane of macrophages, neutrophils and monocytes [390-391] and directly binds to 5-LO [392]. It binds cis-unsaturated fatty acids and is supposed to present AA from cellular membranes to 5-LO, leading to enhanced AA utilization [393-394]. FLAP appears to be necessary for cellular 5-LO activity if AA is produced endogenously [387, 395] but it is dispensable in the presence of exogenous AA [393]. In human leukocytes, the potent FLAP inhibitor MK-886 reduces A23187-induced 5-LO product formation, whereas 5-LO activity in cell-free 5-LO assays is not affected [396].

Coactosin-like protein (CLP) is a 16 kDa-sized protein (142 amino acids) that can bind to human filamentous actin (F-actin) or alternatively to tryptophane residues of the ligand binding loops in the C2-like domain of 5-LO [397-398]. Like 5-LO, CLP is localized in the cytosol of resting cells and translocates to the nuclear membrane upon stimulation [398-399]. CLP enhances the activity of purified 5-LO, increases LTA_4 production in the presence of phosphatidylcholine and the ratio of 5-HETE/5-HPETE [398]. Recent data suggests that CLP also stabilizes 5-LO in terms of preventing non-turnover enzyme inactivation [399].

2.2.5.4 Inhibition of 5-lipoxygenase

For the direct suppression of 5-LO activity, three groups of inhibitors can be distinguished. Redox-active compounds reduce the iron ion in the active site of the enzyme and thereby uncouple its catalytic cycle; many phenolic compounds such as flavonoids are classified in this group [400]. Another group, the iron ligand inhibitors, chelate the active site iron with a hydroxamic acid moiety; BWA4C [401] and zileuton [402] represent members of this group. The third group are the more heterogeneous non-redox type 5-LO inhibitors [25]. High hydroperoxide levels impair the efficiency of these inhibitors, and it was suggested that they compete with AA for binding to the 5-LO's active site [403]. However, hyperforin [404-405] or boswellic acids (BAs) [406] represent non-competitive inhibitors that bind to another site than the AA binding site in the catalytic centre. Another strategy to inhibit 5-LO activity is the inhibition of FLAP. In a cellular environment without exogenous supply of AA, FLAP inhibitors were shown to be effective inhibitors of LT synthesis, e.g. MK-886 offering an IC_{50} value of 2.5 nM in intact neutrophils [25, 407]. Clinically, the direct iron ligand 5-LO inhibitor zileuton and a variety of CysLT1-receptor antagonists (e.g. montelukast) are utilized in the therapy of bronchial asthma [44].

2.2.5.5 12- and 15-lipoxygenases

Generally, the structure of 12-LOs is similar to that of 5-LO. They also consist of a C2-like and a catalytic domain, contain a non-haeme iron in the catalytic centre [408] and translocate to cellular membranes upon stimulation with Ca^{2+} [409-410]. The main product of 12-LO is 12-HPETE that is reduced to 12-HETE [18] or enzymatically converted to hepoxilins [411].

12(R)-LO (epidermis type) was found in the skin of foremost psoriatic patients [412-414] but is also expressed in tonsils [415].

Leukocyte/reticulocyte-type 12/15(S)-LO is abundant in different cells and tissues such as reticulocytes [416], eosinophils [416-417], IL-4-treated monocytes [418-419], the tracheal epithelium [416] and atherosclerotic lesions [46]. 12/15(S)-LO is a rather unspecific enzyme in terms of its substrate specificity and the resulting products as it produces both, 12- and 15-HPETE [408]. Actually, the product profile is critically influenced by the dimension of the active site cavity in 12-LO and 15-LO [420-421]. Regarding the substrates, 12/15(S)-LO is able to convert AA but also other fatty acids such as linolenic acid and linoleic acid and even intact phospholipids [422-425]. 12/15(S)-LO undergoes suicide inactivation within a few minutes of activity [426].

Platelet-type 12(S)-LO is mainly located in platelets and the epidermis [427-428]. In contrast to leukocyte-type 12/15(S)-LO, platelet-type 12(S)-LO restrictively converts AA to the nearly exclusive product 12-HPETE [424]. A distinct feature is the lack of suicide inactivation of the enzyme [426].

Post-translational regulation of 12-LOs appears to be less prominent than in 5-LO as no phosphorylation events or protein interactions have been identified so far. Oxidative stress in human platelets shifts predominant 12-HETE production to the production of anti-inflammatory and anti-thrombotic hepoxilins [429-430].

Besides the leukocyte/reticulocyte-type 12/15(S)-LO, another 15(S)-LO (epidermis-type, or 15-LO-II) is expressed in the human body (skin, cornea); 15-LO-II mRNA was also found in prostate and lung tissue [431-432]. In contrast to the leukocyte/reticulocyte-type 12/15(S)-LO, this enzyme prefers AA to linoleic acid as substrate and exclusively produces 15(S)-H(P)ETE [431].

2.3 Neutrophil proteases

2.3.1 Properties of cathepsin G and human leukocyte elastase

The neutrophil proteases cathepsin G (CG), human leukocyte elastase (HLE) and proteinase 3 (PR3) are serine proteases (30 to 35 kDa) with a conserved catalytic triad (Asp-His-Ser) [3]. The proteins are produced during myeloic maturation of neutrophils and stored in azurophil granules [433-434]. Upon neutrophil stimulation, they are excreted via exocytosis or released into intracellular phagolysosomes [435-436].

2.3.2 Role of neutrophil proteases in inflammation

As delineated in chapter 2.1.2, neutrophils are the first leukocytes to arrive the peripheral tissue from the blood vessels upon stimulation by inflammatory mediators. One of their principal tasks is the neutralization of microorganisms by means of reactive oxygen species, anti-microbial peptides and proteases such as CG, HLE and PR3 [437]. Neutrophil proteases are essential for killing of several microorganisms [435, 438-439], but elevated neutrophil protease levels are also found in inflammatory diseases that lack a microbial background, e.g. in psoriatic lesions [440]. Upon excretion, neutrophil proteases degrade extracellular matrix proteins [441-442], which contributes to tissue destruction in chronic inflammation. Inhibition of neutrophil proteases attenuates the reaction in several *in vivo* models of inflammation, e.g. in collagen-induced arthritis in rats [443-445]. In experimental arthritis, CG or HLE knockout mice exhibit a partial reduction of the inflammatory reaction and animals lacking both enzymes are almost totally resistant [446]. Besides anti-microbial activity and matrix degradation, neutrophil proteases regulate of a multitude of signalling pathways (Figure **5**). They activate or inactivate several cytokines, chemokines and growth factors by cleavage of the respective precursors or the mediators themselves [437]. Furthermore, neutrophil proteases are able to activate cell surface receptors. In platelets, HLE activates αIIbβ3 [447] and CG stimulates the protease activated receptor 4 (PAR4) [448], both of which are associated with enhanced cell aggregation. Further PARs are substrates of neutrophil proteases and cleavage leads to deactivation or activation of these receptors, eliciting multiple effects in various cells [437, 449]. CG is a ligand of the G protein-coupled formyl peptide receptor (FPR), thereby acting as a chemokine on FPR-expressing monocytes and neutrophils [450]. PAR signalling is highly involved in the pathogenesis of arthritis and nociception/pain [451].

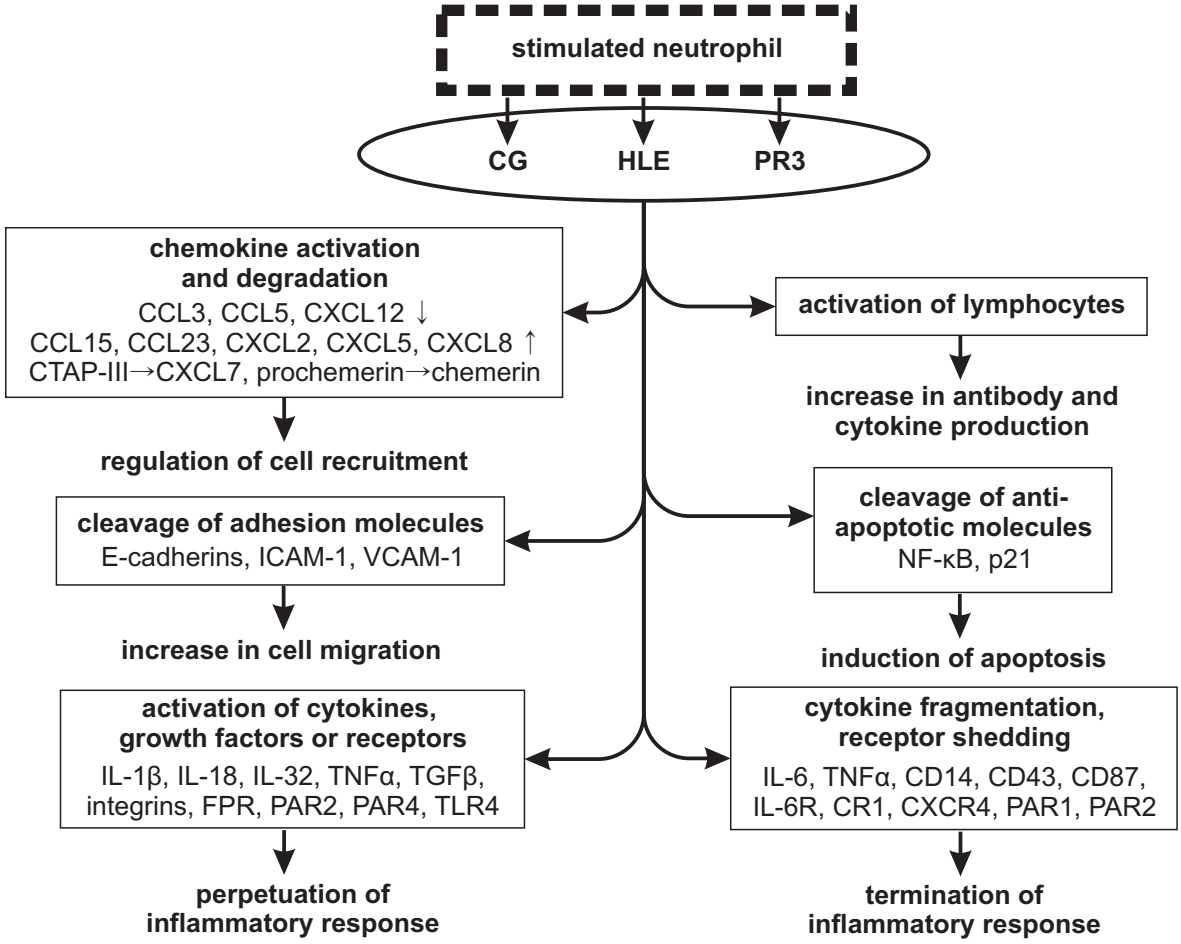


Figure 5: Potential extracellular interactions of neutrophil proteases in inflammatory processes. [437] modified.

2.4 Glucocorticoid signalling

Glucocorticoids are frequently used in the therapy of acute and chronic inflammation. Unlike COX or 5-LO inhibitors, their mode of action cannot be reduced to the inhibition of one single enzyme. They influence several targets leading to both, rapid or delayed anti-inflammatory and immunosuppressive effects [452]. Actually, the molecular mechanism of glucocorticoids is only partly understood and the individual effects are more or less effective depending of the nature of the pro-inflammatory stimulus and the general context [453]. Most of these effects are likely based on the binding of glucocorticoids to the cytosolic glucocorticoid receptor (GR) that is arranged in a protein complex in its resting state [454]. Upon binding of glucocorticoids, the receptor undergoes a conformational change and dissociates from the protein complex [455]. The glucocorticoid-GR complex then translocates to the nucleus [456], where different scenarios may occur; firstly, glucocorticoid-GR complexes may dimerize and bind to glucocorticoid response elements (GREs)

to activate the transcription of responsive genes [456]. The glucocorticoid-induced expression of annexin I and p11/calpactin binding protein for example were reported to lead to inhibited AA release through interaction with $sPLA_2$ and $cPLA_2$ [457-459]. The original hypothesis that these mechanisms were the basis of the eicosanoid-suppressing action of glucocorticoids is no more accepted today [453].

Another way of the glucocorticoid-GR complex to inhibit inflammation is the inhibition of pro-inflammatory transcription factors [460]. This may occur by binding of the complex to negative GREs (nGRE) [461-462], by destabilization of other transcription complexes [463] or by downregulation [464] or direct blockade [465-467] of transcription factors such as NF-κB [465], AP-1 [466], STAT [463-464], nuclear factor of activated T cells (NFAT) [468], GATA-binding protein 3 [461], T-Bet [467] and cAMP response element-binding protein (CREB) [462]. Typical pro-inflammatory target genes of these transcription factors encode the interleukins IL-1β [469], IL-2 [468], IL-5 [470] and IL-6 [469], as well as COX-2 [471], iNOS [472], IFNγ [473], TNFα [474] and the intracellular adhesion molecule (ICAM) [475].

Indirectly, the inhibition of cytokines leads to suppression of other inflammatory mediators or pathways, e.g. to reduced transcription of phospholipases [476]. Moreover, glucocorticoids indirectly suppress the activation of various MAPK (p38, ERK and JNK), e.g. by up-regulation of phosphatases such as MAPK phosphatase 1 (MKP-1), which leads to reduced levels of COX-2, TNFα and IL-1β in LPS-challenged murine macrophages [477]. The glucocorticoid-driven transcription of suppressors of cytokine signalling (SOCS) leads to inhibition of the Janus kinase/STAT (JAK/STAT) pathway [478].

All these mechanisms include genomic interactions of the glucocorticoid-GR complex. Interestingly, glucocorticoid effects are obvious before genomic pathways are able to emerge. Glucocorticoids inhibit smooth muscle contraction within minutes, which is not responsive to the GR antagonist mifepristone [479]. In T cells, the binding of glucocorticoids to their receptor impairs T cell receptor signalling without including genomic mechanisms [480]. G protein-coupled receptor and MAPK signalling were proposed to be implicated in these non-genomic effects [481].

2.5 *Boswellia* species

2.5.1 Botany

Boswellia spec. are sparsely foliated trees that reach up to twelve metres in height [482]. They primarily grow in arid regions of the Horn of Africa, in the very south of the Arabian Peninsula, and in India. The genus *Boswellia* comprises about 20 species and is part of the Burseraceae family. The species that are commonly used for drug recovery are *Boswellia sacra* FLÜCK., which is distributed in the south of Arabia (Oman, Yemen) and the Horn of Africa (where it is traditionally named *Boswellia carteri* BIRDW.), *Boswellia frereana* BIRDW. and *Boswellia papyrifera* HOCHST., which grow in Africa (Ethiopia, Somalia, Eritrea, Sudan, Chad, Nigeria, Cameroon, Uganda and the Central African Republic) and *Boswellia serrata* ROXB., which originates from the northeast of India [482-483].
The resin is collected after incision of the bark; the leaking latex gums on the plant and the solid oleo-gum resin (frankincense) is tapped in intervals of several weeks [484].

2.5.2 Composition of the oleo-gum resins from *Boswellia* species

The oleo-gum resin from *Boswellia* species is composed of an essential oil fraction, a mucilage fraction and a pure resin fraction [483, 485]. The essential oil represents the most diverse fraction, a complex mixture of monoterpenes, sesquiterpenes and diterpenes [486-487]. Its composition is highly dependent on the species and extraction method [488-489]. It makes up five to ten per cent (m/m) of the resin [483, 485] and accounts for the aromatic odour of frankincense. Pharmacological data on this fraction is scarce, but it was found to have anti-microbial [489-490], immunomodulatory [486] and tumour-specific cytotoxic activity [491]. The mucilage fraction amounts up to thirty percent of the oleo-gum resin and comprises polysaccharides composed of monomers such as arabinose and galactose [492]. This fraction was associated with immunomodulatory effects as well [492]. The pure resin fraction makes up about 60% (m/m) of the oleo-gum resin [485]. It consists of several neutral diterpenes but mainly of triterpenes, which feature pentacyclic ursane-, oleanane- or lupane-scaffolds, tetracyclic tirucallane-scaffolds or derivatives thereof [493]. Triterpenic acids usually represent about 50% (m/m) of the pure resin fraction [483, 485]. The quantities of the respective fractions differ depending on environmental fluctuations and the species; e.g. resins from *B. frereana* only contain diminutive amounts of triterpenic acids [494].

Extracts of the oleo-gum resins yielded by extraction with lipophilic solvents (ether, alcohols etc.) contain the pure resin and the essential oil fraction. Evaporation of the solvent partly eliminates the volatile compounds of the essential oil. Thus, these raw extracts contain mostly di- and triterpenes, which can be separated into an acid and a neutral fraction by liquid extraction. The neutral fraction is composed of non-acidic analogues of the triterpenic acids [494] and of diterpenes such as the cembrenes serratol, incensole and incensole acetate [495].

2.5.3 Isolated triterpenic acids from *Boswellia* spec.

Boswellic acids (BAs) represent major ingredients in *Boswellia* spec. oleo-gum resins. They are specific for the genus *Boswellia* and reach 14 to 25% (m/m) of the lipophilic extract from *B. serrata* oleo-gum resin [496-498]. BAs offer two pentacyclic triterpene scaffolds that differ in the constitution of the methyl groups in position 19 and 20; the oleanane scaffold is the basal structure of α-constituted BAs and the ursane scaffold originates the β-constituted BAs [497]. Derivatization of the skeletal structures at position 3 and oxidation of C-11 bring forth the acetyl- and keto-analogues, leading to β-boswellic acid (β-BA), 11-keto-β-boswellic acid (KBA), 3-*O*-acetyl-β-boswellic acid (A-BA), 3-*O*-acetyl-11-keto-β-boswellic acid (AKBA) and the respective α-constituted derivatives α-boswellic acid (α-BA), 11-keto-α-boswellic acid (K-α-BA), 3-*O*-acetyl-α-boswellic acid (A-α-BA) and 3-*O*-acetyl-11-keto-α-boswellic acid (AK-α-BA) (Figure **6**).

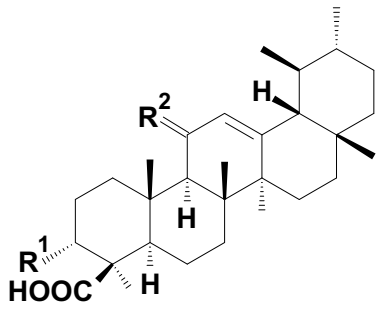
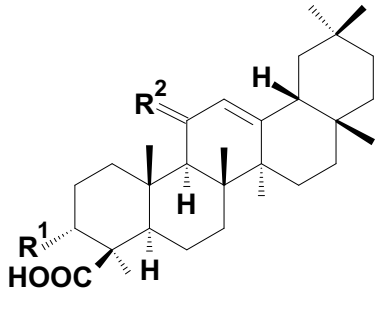
	R^1	R^2	name
	HO	H, H	β-BA
	HO	O	K-BA
	O, O	H, H	A-BA
	O, O	O	AKBA
	HO	H, H	α-BA
	HO	O	K-α-BA
	O, O	H, H	A-α-BA
	O, O	O	AK-α-BA

Figure 6: Molecular structures of boswellic acids.

Nyctanthic acids (NAs) and roburic acids (RAs) represent *seco*-derivatives of α- and β-constituted BAs that exhibit an open A-ring. Roburic acid (RA), 4(23)-dihydro-roburic acid (DH-RA), 4(23)-dihydro-11-keto-roburic acid (DH-k-RA) and 4(23)-dihydro-nyctanthic acid (DH-NA) (Figure **7**) were isolated as minor components from *B. carteri* [499-500].

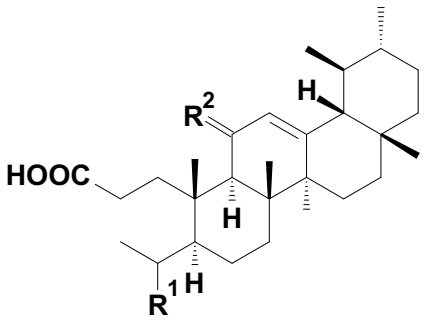
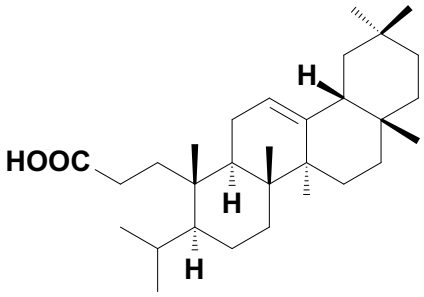
	R^1	R^2	name
	CH_2	H, H	RA
	CH_3	H, H	DH-RA
	CH_3	O	DH-k-RA
			DH-NH

Figure 7: Molecular structures of roburic and nyctanthic acids.

Lupeolic acid (LA) was found in “African” frankincense [501] and the derivative 3-*O*-acetyl-lupeolic acid (Ac-LA) was originally isolated from *B. serrata* resin [502] (Figure **8**). In methanolic extracts from frankincense, these pentacyclic triterpenic acids represent minor components with less than 1% (m/m), respectively [497]. Recently, a derivative of these lupeolic acids (LAs) was isolated from *B. carteri* that offers a primary hydroxy-function in position C-28, namely 3-*O*-acetyl-28-hydroxy-lupeolic acid (Ac-OH-LA) [500].

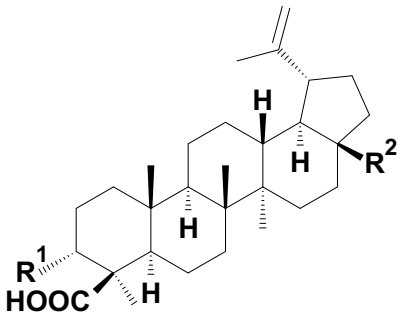
	R^1	R^2	name
	HO	CH_3	LA
	O, O	CH_3	Ac-LA
	O, O	OH	Ac-OH-LA

Figure 8: Molecular structures of lupeolic acids.

Furthermore, *Boswellia* spec. comprise tetracyclic tirucallic acids (TAs) [503-504]. It should be noted that TAs or derivatives thereof are also part of further resinous drugs such as from *Canarium* [505], *Protium* [506] and *Pistacia* species [507]. TA derivatives mainly differ at the C-3 carbon depending of the oxidation state (secondary alcohol or ketone), the configuration of the contingent hydroxy-group and the acetylation of this moiety. Further derivatives arise from the positioning of the cyclic double bond that is located in position 7 or 8, leading to the derivatives 3-α-hydroxy-8,24-dien-tirucallic acid (3α-OH-8,24-dien-TA), 3α-*O*-acetyl-8,24-dien-tirucallic acid (3α-Ac-8,24-dien-TA or Ac-TA), 3-β-hydroxy-8,24-dien-tirucallic acid (3β-OH-8,24-dien-TA or 3β-OH-TA) and 3-oxo-8,24-dien-tirucallic acid (3-oxo-TA), 3-α-hydroxy-7,24-dien-tirucallic acid (3α-OH-7,24-dien-TA or 3α-OH-TA) and 3α-*O*-acetyl-7,24-dien-tirucallic acid (3α-Ac-7,24-dien-TA) (alternative abbreviations are used in chapter 4.2 for simplification reasons, Figure **9**). Resins from *B. papyrifera* and *B. serrata* contain considerable amounts of TAs, especially 3-oxo-TA (Table **2**).

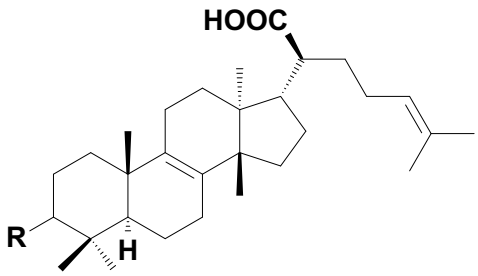
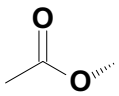
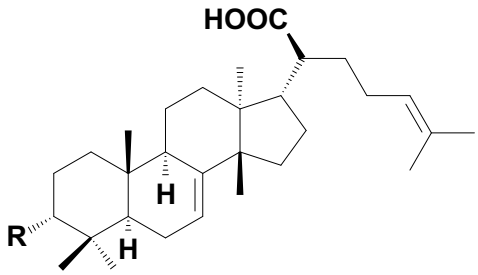
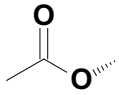
	R	name
	HO	3α-OH-8,24-dien-TA
		3α-Ac-8,24-dien-TA
	HO	3β-OH-8,24-dien-TA
	O	3-oxo-TA
	HO	3α-OH-7,24-dien-TA
		3α-Ac-7,24-dien-TA

Figure 9: Molecular structures of tirucallic acids.

The contents of various compounds in the oleo-gum resin of *B. papyrifera* and *B. serrata* were quantified by M. Paul (University of Saarland, Saarbrücken) as part of a current research cooperation (Table **2**).

compound	*B. papyrifera* [% (m/m)]	*B. serrata* [% (m/m)]
α-BA	0.936	1.58
A-α-BA	1.75	0.912
β-BA	1.32	4.08
A-BA	3.13	3.17
K-BA	0.276	0.337
AKBA	3.90	0.570
α-OH-8,24-dien-TA	0.544	1.34
β-OH-8,24-dien-TA	0.650	1.12
3-oxo-TA	2.23	2.64
3α-Ac-8,24-dien-TA	0.829	0.848
Ac-LA	0.454	0.208

Table 2: Content of the major triterpenic acids in oleo-gum resins from B. papyrifera and B. serrata. According to [498]. Amounts of the triterpenic acids after exhaustive Soxhlet extraction with diethyl ether of the oleo-gum resin from *B. papyrifera* or *B. serrata* are given as percentage (m/m) of the crude oleo-gum resin. Compounds were quantified by HPLC analysis of the extracts as described in the methods. The areas under the peaks were referred to calibration curves generated with the isolated compounds.

2.5.4 Medical use of *Boswellia* preparations

2.5.4.1 Traditional use

Frankincense was already used as a herbal remedy in the ancient cultures of Egypt, Rome and the traditional medicine of the orient, China and India, with a focus on treatment of inflammation (arthritis, ulcers, skin diseases, fever, diabetes) [508-509]. Frankincense was listed in the German pharmacopoeia (DAB 1, 1872) and was used in the therapy of huskiness, abscesses and inflammations of the mouth, throat and ovaries [510]. With the emergence of synthetic drugs in modern medicine attention for phyto-pharmaceuticals vanished, manifest in the last entry of frankincense (or “Olibanum”) in the German pharmacopoeia in 1941. With the upcoming attention for alternative medicine, interest for the resin resurrected and frankincense re-appears in the European pharmacopoeia (Ph. Eur.) since edition 5.7.

2.5.4.2 Clinical effectiveness and safety data of frankincense formulations

Clinical data on frankincense formulations include pilot studies on arthritis, inflammatory bowel diseases, bronchial asthma and cancer, all of which are based on or associated with a chronic inflammatory background [508] (Table **3**). On the other hand, the focus on chronic inflammation might result from convenient patient recruitment. Experiments in animal models suggest efficacy in some models of acute inflammation as well [511-512].

Table 3: Clinical studies on frankincense formulations. H15® lipophilic *B. serrata* extract, WokVel™ *B. serrata* extract standardized on 40% BAs, 5-Loxin® *B. serrata* extract standardized on 30% AKBA, Aflapin® *B. serrata* extract enriched in AKBA and non-volatile oil, "S-Compound" *B. serrata* preparation, MMP-3 matrix metalloproteinase 3.

Disease	Study design	Outcome → comments
Rheumatoid arthritis [513]	Meta- analysis of 11 studies; H15®	Reduction of pain and swelling of joints → Inhomogeneous study-design and lack of transparency void proper assessment
[514]	Placebo controlled; H15® (3,600 mg/d for 12 weeks); 18 patients (verum), 19 patients (placebo)	No significant beneficial effects on pain, swelling, blood sedimentation, c-reactive protein level, requirement of additional NSAIDs → Small number of patients and concomitant treatment with glucocorticoids and disease-modifying anti-rheumatic drugs limit the power of the outcome
Osteo-arthritis [515]	Placebo controlled, crossed-over; WokVel™ (999 mg/d for 8 weeks); 30 patients	Reduction of pain and swelling of arthritic knees, increased knee flexion and walking distance, unchanged radiological results
[516]	Prospective, comparison of WokVel™ (999 mg/d) and valdecoxib (10 mg/d); 33 patients per group, 6 months	Improvement of pain, stiffness and difficulty in performing daily activities in both groups → Effect appeared faster in valdecoxib group (1 month vs. 2 months in WokVel™ group) but relapsed directly after abortion of the therapy, whereas WokVel™ effect persisted for ≥ 1 month
[517]	Randomized, placebo-controlled 5-Loxin® (100 and 250 mg/d for 90 d); 25 patients per group	Dose-dependent amelioration of pain, physical functions and MMP-3 levels in the synovial fluid
[518]	Placebo-controlled, comparison of 5-Loxin® and Aflapin® (both 100 mg/d for 90 d); 20 patients per group	Dose-dependent amelioration of pain, physical functions and MMP-3 levels in the synovial fluid in both verum groups with superiority of the Aflapin® group
Morbus Crohn [519]	Double-blind, comparison of H15® (3,600 mg/d, 44 patients) and mesalazine (4,500 mg/d, 39 patients), 8 weeks	Comparable efficacy of both treatments with respect to the Crohn disease activity index (CDAI) accompanied with superior tolerance of H15®
Ulcerative colitis [520]	Comparison of pulverized *B. serrata* resin (standardized on 1.8% KBA and 1.4% AKBA, 1,050 mg/d, 34 patients) with sulfasalazine (3,000 mg/d, 8 patients) , 6 weeks	Improvement of all tested parameters (stool properties, histopathology, haemoglobin, serum iron, calcium, phosphorus, proteins and total leukocyte and eosinophil counts) in both groups, remission of 82% and 75% of the patients in the *Boswellia*- and sulfasalazine-treated group, respectively
Chronic colitis [521]	Comparison of "S-Compound" (900 mg/d, 20 patients) with sulfasalazine (3,000 mg/d, 10 patients), 6 weeks	One or more of the parameters (stool properties, histopathology, haemoglobin, serum iron, calcium, phosphorus, proteins and total leukocyte and eosinophil counts) improved in 90% and 60% of the "S-Compound" and sulfasalazine group, respectively, with 70% and 40% of the patients going into remission, respectively
Collagenous colitis [522]	Double-blind, placebo-controlled, *B. serrata* extract (standardized on 80% acidic compounds, 1,200 mg/d, 6 weeks) , 11 patients (verum), 15 patients (placebo)	Remission of 64% of the patients in the verum group vs. 27% in the placebo group
Bronchial asthma [523]	Double-blind, placebo-controlled, "S-Compound" (900 mg/d, 6 weeks) , 40 patients in both groups	Improvement of the test parameters (dyspnoea, rhonchi, numbers of attacks, different lung function tests) in 70% of the "S-Compound"-treated patients vs. 27% in the control group

Several clinical studies using H15® were performed in patients suffering from intra-cranial tumours [524-526]. A direct anti-proliferative effect could not be confirmed, but improvement of intra-cranial oedema and related symptoms was observed. Nevertheless, small groups of patients, parallel medication and the lack of controls complicate the assessment of these studies.

Regarding the toxicological aspects of *Boswellia* preparations, only minor evidence of side effects has been reported in therapeutic dosage. In dosages exceeding therapeutic dimensions, no genotoxic activity of BAs (at 1,000 mg/kg) [527] or sub-chronic toxicity of *B. papyrifera* resin or 5-Loxin® extract were found in rats [528-529]. Extremely high concentrations of *B. papyrifera* resin (1,000 mg/(kg × d) for 28 days or single administration of 5,000 mg/(kg × d)) led to toxic effects such as decreased body weight, weakness, reduced motion activity and variations of some of haematological and biochemical parameters in rats [528]. A study analysing the toxicity of the extract 5-Loxin® in rats found LD_{50}s greater than 5,000 mg/kg (peroral) and 2,000 mg/kg (dermal) [529].

In an aforementioned clinical study using H15®, substantial laboratory parameters were registered for the evaluation of toxicological effects (haemogram, creatinine, alkaline phosphatase, γ-glutamyl transpeptidase, glutamic-pyruvic transaminase, urinalysis) [514]. None of these parameters was abnormally changed during the therapy [514]. Some of the clinical studies documented the occurrence of gastrointestinal disorders (nausea, abdominal pain and cramping, diarrhoea, pyrosis) [346, 515-516, 521-522]. A causative association of these side effects and the therapy with frankincense formulations could not definitely be passed because of the small number of events. In summary, frankincense formulations are promising tools in the therapy of inflammatory diseases featuring a favourable safety profile.

2.5.4.3 Molecular mechanisms affected by compounds isolated from *Boswellia* preparations

Traditional medicine and modern studies support the anti-inflammatory potential of frankincense formulations. Several molecular targets possibly underlying these effects have been identified in the last decades.

The first target to be identified for frankincense extracts and BAs was 5-LO [530-531]. AKBA was found to be the most potent BA with an IC_{50} of 1.5 µM in A23187-stimulated rat peritoneal PMNL and the BAs turned out to be non-redox type inhibitors of 5-LO [531]. However, the IC_{50} values for AKBA deviate markedly (1.5 – 50 µM) depending on the assay conditions [406, 532]. Additional interference with cellular pathways may account for enhanced potency of BAs in some cellular systems. The carboxylic group in ring A and the keto-function in the C-11 position enhance the 5-LO inhibitory potential. Non-inhibitory derivatives (e.g. ursolic acid, amyrin) are able to compete with AKBA for the binding site on 5-LO, which is different from the catalytic site [406, 533]. Binding of AKBA is Ca^{2+}-dependent and competes with high concentrations of AA, suggesting a common allosteric binding site [534].

In a cellular context, frankincense formulations exhibit stimulatory effects on 5-LO activity as well [535]. Ethanolic extracts enhanced A23187-induced 5-LO product formation in PMNL in concentrations below 5 µg/ml, whereas product formation is inhibited at higher concentrations. AKBA itself only performs inhibitory effects in stimulated neutrophils. In resting PMNL, AKBA and KBA induce p38 and ERK-1/-2 MAPK activation, Ca^{2+} mobilization and the formation of reactive oxygen species, which leads to AA release and enhanced 5-LO product formation [536-537]. Similar to frankincense extracts, the tetracyclic triterpene 3-oxo-TA stimulates A23187-induced 5-LO product formation in PMNL at concentrations below 10 µM and acts inhibitory at concentrations above 10 µM [538]. In contrast, its derivative 3α-OH-8,24-dien-TA constantly acts inhibitory. 3-oxo-TA (but barely 3α-OH-8,24-dien-TA) induces MAPK/ERK kinase-1/-2 (MEK-1/-2) phosphorylation, which is associated with enhanced translocation of 5-LO to the membrane compartment of the cell.

Although BAs were primarily claimed to be exclusive inhibitors of 5-LO not affecting other enzymes of AA metabolism such as 12-LO and COX [531], inhibition of platelet-type 12-LO (p12-LO) [539] and COX-1 [540] was found in more recent analyses. Both COX-1 and p12-LO bind directly to immobilized BAs. COX-1 activity is inhibited by BAs in stimulated platelets with AKBA being the most potent compound (IC_{50} ~ 6 µM), and all tested BAs inhibited COX-1 (IC_{50} ~ 32 µM) in a cell-free assay [540]. The inhibition is reversible, competes with AA, and binding to the catalytic site was reproduced in an automated docking approach. Binding and inhibition of COX-2 is by far less prominent. AKBA (IC_{50} ~ 15 µM) and β-BA inhibit p12-LO in cell-free assays, but BAs stimulate 12-HETE production in platelets [539]. The stimulatory effect is preserved after deprivation of Ca^{2+} and is caused by $cPLA_2$α-dependent AA supply, which, in the presence of Ca^{2+}, is associated with PI3 and Src kinase activity [539, 541].

Most recently, the microsomal PGE_2 synthase-1 (mPGES-1) was found to bind immobilized BAs in a target fishing approach [540]. Direct binding was confirmed by surface plasmon resonance spectroscopy and led to suppression of mPGES-1 activity with IC_{50} values of 3 to 10 µM in a cell-free assay. PGE_2 synthesis was also inhibited in stimulated A549 cells and LPS-challenged human blood, without impact on other COX-dependent metabolites. Carrageenan-induced pleurisy in rats and paw oedema in mice were inhibited by BAs, which was accompanied by reduced PGE_2 formation. β-BA was the most potent BA causing significant inhibition of pleurisy and PGE_2 formation after peroral administration of 1 mg/kg.

Another molecular target of BAs is human leukocyte elastase (HLE) with an IC_{50} of 15 µM for AKBA in a cell-free assay [542]. The inhibition of HLE by triterpenic acids was already shown for ursolic acid (IC_{50} ~ 2 µM) and even for neutral triterpenes like amyrin [542-543].

Recently, direct interaction of BAs with cathepsin G (CG) was demonstrated in a target fishing approach [544]. Automated docking suggests tight binding to the same site as an established CG inhibitor. Proteolytic activity of CG is inhibited at sub-micromolar concentrations of β-BA, AKBA and A-BA and functional effects of CG (chemoinvasion in matrigel, Ca^{2+} mobilization in platelets) are reduced upon treatment of neutrophils with BAs. Oral administration of frankincense extracts in a clinical study even inhibited CG activity in human blood. These results are in line with earlier observations that found the inhibition of inflammatory leukocyte migration in rats after treatment with frankincense extracts [545].

A-α-BA and AKBA inhibit IκB kinases α and β (IKK), suppress the phosphorylation of IκBα and p65 and thus hinder the translocation of the NF-κB complex to the nucleus [546]. This was associated with the suppression of LPS-stimulated induction of TNFα in human monocytes at BA concentrations in the low micromolar range.

Further reports indicate interactions of frankincense preparations and BAs with the immune system, such as the inhibition of the C3-convertase [547], stimulation of mitogen-induced lymphocyte proliferation [548] and enhanced release of Th2-derived cytokines (IL-4, IL-10) along with reduced Th1-derived (IFNγ, IL-2) cytokine production in stimulated lymphocytes [549]. A recent study demonstrated the interference of AKBA with STAT3 signalling by induction of Src homology region 2 domain containing phosphatase 1 (SHP-1) [550]. The resulting inhibition of IL-6-induced transcription through STAT3 was associated with reduced proliferation of cancer cells [550] but might also inhibit the induction of pro-inflammatory cytokines such as IL-17 and IL-23 that are highly involved in chronification of inflammation [551].

Many studies have been performed on anti-proliferative, pro-apoptotic and cell-differentiating effects of frankincense formulations (reviewed in [512]). Interaction of BAs (especially 3-*O*-acetyl-derivatives) with topoisomerases I and IIα may partly account for these actions (IC_{50} values of 1 to 30 µM) [552-553]. Moreover, inhibition of IKK was shown to contribute to cell death and inhibition of prostate cancer cell proliferation [554]. As ERK is part of anti-apoptotic signalling in many cancer cells [555], inhibition of this kinase by AKBA may account for anti-proliferative effects as well [556].

Data on molecular targets of *Boswellia*-derived terpenes besides BAs is quite scarce. TA-induced MEK-driven activation of 5-LO activity in PMNL was already mentioned above [538]. Furthermore, TAs directly inhibit Akt1 and Akt2 (IC_{50} values of 0.1-1 µM for 3β-Ac-8,24-dien-TA), which induces apoptosis of prostate cancer cells that exhibit constitutive Akt activity without affecting the NF-κB pathway or the viability of non-cancerous cells [557].

The extract of the gum resin from *B. frereana*, which contains diminutive amounts of acids, was shown to suppress MMP activation, as well as NO and PGE_2 production in stimulated cartilage tissue, which was attributed to the major component lupeol [558]. The neutral compounds incensole and incensole acetate, which represent major components of the neutral fraction from *B. papyrifera* [488], are recent subjects of investigation. Both substances suppress TNFα- or LPS-stimulated activation of the NF-κB pathway by inhibition of IKK phosphorylation [559]. Significant inhibition of carrageenan-induced paw oedema in mice was shown for incensole acetate. However, the applied dose was relatively high (50 mg/kg) and so was the IC_{50} for TNFα-induced NF-κB activation in Jurkat cells (50 µM). Incensole acetate significantly inhibits the LPS-induced NF-κB-mediated production of pro-inflammatory mediators (TNFα, IL-1β, IL-6 and PGE_2) in human monocytes with IC_{50} values of 15 to 30 µM [560].

2.5.4.4 Pharmacokinetics

Pharmacokinetic parameters are crucial for assessment of the final efficacy of drugs *in vivo*, but data so far are only available for BAs. Transfer of these data to other triterpenic acids might serve as vague reference. Single dose application of 333 mg WokVel^{TM} extract (*B. serrata*) peaks in plasma concentrations of about 3 µM KBA after 4.5 h and the elimination half-life is about 6 h [561]. Single administration of 1,600 mg of another *B. serrata* extract results in KBA peak concentrations of 1.7 µM within 1 h whereas AKBA is not detected [562]. Repeated administration of four daily doses of 786 mg extract (*B. serrata*) for 10 days leads to plasma levels of 10 µM β-BA, 2.4 µM A-BA, 0.34 µM KBA and 0.1 µM AKBA [563] which is approximately in line with another study [544].

Food intake critically influences bioavailability of BAs. Peak plasma levels are increased 3- to 6-fold when *B. serrata* extracts are administered with a high fat diet instead of fasted conditions [564]. Non-acetylated BAs are intensively oxidized within phase I metabolization whereas the acetylated derivatives are poorly transformed [565]. No phase I metabolization (including deacetylation) was observed for AKBA *in vivo*. Restricted permeability in Caco-2 models provides an explanation for the relatively poor bioavailability of 11-keto-BAs [566]. Moreover, 11-keto-BAs interact with the organic anion transporter OATP1B3 and multi-drug resistance protein 2 (MRP2) but not with P-glycoprotein. Frankincense extracts from different *B. species* inhibit diverse cytochrome P450 enzymes, with BAs contributing to inhibition (IC_{50} values of 5-100 µM) but undefined compounds being the major inhibitors [567].

2.6 Aim of this work

Extracts from the oleo-gum resin of *Boswellia* species have been used in the treatment of diverse diseases since ancient times [508]. In modern medicine, frankincense formulations first fell into oblivion but re-emerged in the 1980s with upcoming data supporting their therapeutic efficacy accompanied by exiguous toxicity. Recent clinical studies and experiments in animal models stated the therapeutic efficacy of frankincense formulations in inflammatory diseases such as osteoarthritis, inflammatory bowel diseases, bronchial asthma and cancer [508, 512]. From the beginning of modern investigation of the resin, a pivotal role was attributed to the genus-specific BAs. Several molecular targets of these compounds have been identified so far: 5-LO [530-531], platelet-type 12-LO [539], COX-1 [540], mPGES-1 [511], CG [544], HLE [542] and IκB kinase [546]. BAs represent a considerable part of the resin but other compounds, which make up about 85% of the whole oleo-gum resin or about 50% of the acid fraction [483, 497] were neglected in former investigations. So far, only TAs were rudimentarily investigated and were shown to modulate 5-LO product formation in cellular models [538]. Furthermore, incensole and incensole acetate were demonstrated to interact with the NF-κB pathway [559]. The molecular targets identified for BAs, incensole and incensole acetate provide some explanation for the clinical efficacy of frankincense formulations. However, for some of these targets, the IC_{50} values are relatively high and the realization of the required plasma levels after administration of reasonable doses of frankincense extracts is uncertain. Moreover, BAs only represent a more or less random selection of the plethora of structurally diverse compounds comprised in the oleo-gum resin and thus might only contribute in part to the overall anti-inflammatory activity.

This work is part of a joined project with the University of Saarland (Michael Paul, Johann Jauch) and the Aureliasan GmbH (Tübingen, Germany). The project is supposed to reveal the composition of frankincense extracts derived from different *Boswellia* species, to identify pharmacologically active principles besides BAs, and to evaluate the contribution of the individual compounds to the overall biological effects of extracts. The objective is to provide the raw material and extraction methods that yield an extract with optimized activity.

The aim of the present part of this project is the pharmacological characterization of frankincense-derived triterpenic acids besides BAs. Their impact on targets of BAs is assessed and compared to the results obtained for BAs. $cPLA_2\alpha$, 5-LO, COX-1 and -2, mPGES-1, CG, HLE and the glucocorticoid-glucocorticoid receptor interaction are central subjects of investigation.

3 Materials and Methods

3.1 Materials

[5,6,8,9,11,12,14,15-^{3}H]-AA and MK-886 were from BIOTREND GmbH (Cologne, Germany), p-anisidinium chloride, EDC (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride), cPLA$_2\alpha$ inhibitor (RSC-3388), JNJ-10311795, KN-62, KN-93, N-methoxysuccinyl (MeOSuc)-Ala-Ala-Pro-Val-p-nitroanilide (human leukocyte elastase substrate), sivelestat and U46619 were from Calbiochem/Merck KGaA (Darmstadt, Germany) and BAPTA-AM (1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester)), CDC (cinnamyl-3,4-dihydroxy-α-cyanocinnamate), fMLP, Fura-2-AM (1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methyl-phenoxy) ethane-N,N,N',N'-tetraacetic acid, pentaacetoxymethyl ester), okadaic acid and U0126 were purchased from Enzo life sciences GmbH (Lörrach, Germany). BSA (bovine serum albumin), cathepsin G (human, purified), EDTA (ethylenediaminetetraacetate, disodium salt dihydrate) and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid) were from Applichem GmbH (Darmstadt, Germany), COX-1 (ovine), COX-2 (human, recombinant) and 11β-PGE$_2$ were from Cayman Chemical Company (Ann Arbor, MI, USA) and DMSO (dimethylsulphoxide), β-mercaptoethanol, sodium dodecyl sulphate (SDS), Spectra/Por® Regenerated Cellulose Dialysis Membrane (Cut-off 25,000) and Tween 20 were obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). ATP was from Roche Diagnostics GmbH (Mannheim, Germany), Hybond™ ECL membrane was from GE Healthcare Europe GmbH (Munich, Germany), indomethacin was from Fagron GmbH (Barsbüttel, Germany), collagen (Kollagenreagenz Horm®) from Nycomed Pharma GmbH (Wien, Austria), Ni-NTA agarose from Qiagen GmbH (Hilden, Germany), peqGold Protein Marker IV from peqLab Biotechnology GmbH (Erlangen, Germany), PGH$_2$ from Larodan Fine Chemicals (Malmö, Sweden), SureFECT™ from SABiosciences corp. (Frederick, MD, USA) and Ultima Gold™ XR was from Perkin Elmer Inc. (Boston, MA, USA). λ-Carrageenan type IV was from Sigma-Aldrich S. r. l. (Milan, Italy) and AA, A23187, cholesterol, cytochalasin B, 2,7-dichlorofluorescin diacetate, essentially fatty acid-free BSA (FAF-BSA), γ-linolenic acid, LPS (from *Escherichia coli* 026:B6), N-Suc-Ala-Ala-Pro-Phe-p-nitroanilide (cathepsin G substrate), PGB$_1$ and thrombin were obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany), as well as all other chemicals, which are not mentioned separately.

BWA4C, CV-4151, SB203580 and MD-52 were generous gifts by Dr. L. G. Garland (Wellcome Research Laboratories, Kent, UK), Prof. Dr. S. Laufer (University of Tübingen, Germany) and Prof. Dr. M. Schubert-Zsilavecz (University of Frankfurt, Germany), respectively.

Anti-cPLA$_2$-antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), anti-β-actin-, anti-phospho-Akt-, anti-phospho-Ser^{505}-cPLA$_2$-, anti-phospho-p44/42 MAPK-, anti-phospho-JNK- and anti-phospho-p38 MAPK-antibodies were from Cell Signaling Technology Inc. (Danvers, MA, USA). Secondary antibodies were from Sigma-Aldrich Chemie GmbH (Munich, Germany) (alkaline phosphatase- and peroxidase-coupled antibodies) and GE Healthcare Europe GmbH (Munich, Germany) (fluorescent dye-coupled antibodies).

PGE$_2$- and 6-keto PGF$_{1\alpha}$-ELISA kits were purchased from Assay Designs Inc. (Ann Arbor, MI, USA) and LTB$_4$- and 6-keto PGF$_{1\alpha}$-ELISA kits for *in vivo* experiments were from Cayman Chemical Company (Ann Arbor, MI, USA). The PGE$_2$ RIA used for *in vivo* experiments was from Sigma-Aldrich S. r. l. (Milan, Italy). The Cignal^{TM} GRE Reporter Assay Kit was provided by SABiosciences corp. (Frederick, MD, USA) and the dual-luciferase reporter assay system was from Promega GmbH (Mannheim, Germany).

All cell culture media, LSM 1077 (Lymphocyte Separation Medium), trypsin/EDTA solution, glutamine, penicillin and streptomycin were from PAA Laboratories GmbH (Coelbe, Germany). All lipids for non-cellular cPLA$_2\alpha$ assays (1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phospho-ethanolamine (PAPE), 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC) and 1-palmitoyl-2-oleoyl-*sn*-glycerol (POG)) were from Avanti Polar Lipids Inc. (Alabaster, AL, USA). High performance liquid chromatography (HPLC) solvents were from Merck KGaA (Darmstadt, Germany). All plastic ware was from Greiner Bio-One GmbH (Frickenhausen, Germany).

3.2 Extraction of *Boswellia* oleo-gum resins and separation in fractions

The oleo-gum resins of *Boswellia* species were powdered, filled in cellulose tubes and extracted with dichloromethane in a Soxhlet extractor for 16 h. Removal of the solvent led to the raw extract (RE). For separation of the acidic compounds, RE was dissolved in diethyl ether and alkalized with potash lye (5%, m/v). The mixture was shaken in a separating funnel and the aqueous phase was washed thrice with diethyl ether. The ether phases were combined, washed with saturated common salt solution and dried with anhydrous magnesium sulphate. Evaporation of the ether yielded the neutral fraction (n. f.). After acidification of the potash lye-phase with hydrochloric acid to pH 2-3, the aqueous phase was extracted thrice with diethyl ether. The pooled organic phases were washed with saturated common salt solution and dried with anhydrous magnesium sulphate. Evaporation of the ether resulted in the acid fraction (a. f.).

3.3 Fractionation of extracts by flash chromatography

Flash chromatography of extracts from the oleo-gum resin of *Boswellia* species was performed by Dr. S. Seitz and M. Paul (University of Saarland, Saarbrücken, Germany).

The stationary phase (normal phase silica gel, particle size 40-63 µm, 1,400 ml, Merck KGaA, Darmstadt, Germany) was equilibrated in mobile phase (pentane-diethyl ether (8:1, v/v) and acetic acid 1%, v/v), degassed, and filled in a glass column (10 cm). The sample was dissolved in the appropriate amount of mobile phase and filled on the chromatographic bed. Elution was carried out by application of 1 bar and usage of a pentane-diethyl ether gradient from 8:1 to 1:2 (v/v). Fractions were collected each 10 sec. The fractions were analyzed by thin layer chromatography (silica gel 60, F_{254}, Merck KGaA, Darmstadt, Germany, pentane-diethyl ether (2:1, v/v) plus acetic acid 1%, v/v) and fractions containing the same compounds were merged.

3.4 Analysis of extracts by HPLC and structure elucidation of isolated compounds by MS and NMR

Analysis of extracts from the *Boswellia* oleo-gum resins by HPLC and structure elucidation of isolated compounds by mass spectrometry (MS) and nuclear magnetic resonance (NMR) were performed by Dr. S. Seitz and M. Paul (University of Saarland, Saarbrücken, Germany).

Analytical systems: The acid fraction (see chapter 3.2) and its subfractions (see chapter 3.3) were analyzed on a Nucleodur® C18 ec-column (250 × 4 mm, 5 µm particle size, Macherey & Nagel, Düren, Germany) or a YMC-Pack Pro C18 RS-column (250 × 4.6 mm, 5 µm particle size, YMC Co., Ltd., Kyoto, Japan), using a gradient from 85 to 100% methanol with 0.1% (v/v) trifluoroacetic acid (0.85 ml/min).

Preparative systems: Isolated acids were obtained by preparative chromatography using a Nucleodur® C18 ec-column (250 × 21 mm, 5 µm particle size, Macherey & Nagel, Düren, Germany) or a YMC-Pack Pro C18 RS-column (250 × 20 mm, 5 µm particle size, YMC Co., Ltd., Kyoto, Japan), using a gradient from 85 to 100% methanol (23.4 ml/min).

The separated compounds were detected by UV-detection at 210 nm, collected and characterized by MS (MAT 95 S (Bruker, Karlsruhe, Germany) and ZQ4000-ESI-MS (single quadrupole, Waters, Milford, MA, USA)) and NMR-spectroscopy (^{1}H, ^{13}C, DEPT 90, DEPT135, H, H-COSY, HMQC, HMBC, HMQC-COSY and NOESY, AV II 400 and AV 500-devices (Bruker, Karlsruhe, Germany)). The purity of the isolated compounds was > 98% as determined by DAD-HPLC.

3.5 Cells

A549 cells

A549 cells (human lung epithelial cell line) were obtained from Dr. O. Rådmark (Karolinska Institute, Stockholm, Sweden). HERAcell incubators (Thermo Fisher Scientific Inc., Langenselbold, Germany) were used (37 °C, 6% CO_2) for culturing. Cells were cultured in DMEM/high glucose (4.5 g/l) medium supplemented with FCS (10%, v/v), penicillin (100 U/ml) and streptomycin (100 µg/ml). Confluent cells were split every 3 days after detachment with trypsin (0.5 mg/ml) / EDTA (0.22 mg/ml) solution and seeded out at 2×10^6 cells in 20 ml medium.

Sf9 cells

Insect cells from *Spodoptera frugiperda* (Sf9 cells) were obtained from Prof. Dr. J. Z. Haeggström (Karolinska Institute, Stockholm, Sweden). Cells were cultured at 27 °C in an incubator (Binder GmbH, Tuttlingen, Germany) in Erlenmeyer flasks under continuous stirring. For culturing, Insect Express Sf9-S2 medium that was supplemented with 10% FCS, penicillin (100 U/ml) and streptomycin (100 µg/ml) was used. Cells were split when the cell density exceeded 2×10^6 cells per ml and seeded out at 5×10^5 cells per ml.

Human blood cells

Blood cells were isolated from leukocyte concentrates (buffy coats) from the blood centre University Hospital Tübingen (Germany). Venous blood from healthy donors that did not take any medication for at least 7 days was taken and centrifuged at 4,000 × g, 20 min at room temperature (RT). The cell concentrate was then diluted with cold phosphate buffered saline (PBS) buffer (1:1, v/v). Erythrocytes were separated by dextran sedimentation (dextran solution 5% (m/v in PBS) was mixed with the cell suspension in a ratio of 1:4, v/v) for 30 min and the leukocyte concentrate was layered on LSM 1077 (leukocyte separation medium) cushions and centrifuged at 1,000 × g, 10 min at RT.

Pelleted PMNL were purified from resting erythrocytes by hypotonic lysis as described [383] and resuspended in the appropriate volume of PBS buffer supplemented with 1 mg/ml glucose (PG buffer, purity > 96-97%).

Peripheral blood mononuclear cells (PBMC) were collected after density gradient centrifugation, washed thrice with cold PBS buffer and resuspended in PG buffer in the indicated density.

Monocytes were isolated from PBMC, which were resuspended in RPMI 1640 medium supplemented with 2 mM glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin and 10% FCS and spread (2×10^7 cells /ml) in cell culture flasks for 2 h at 37 °C, 6% CO_2. Suspended lymphocytes were removed by suction and repeated washing with PBS buffer. Adherent monocytes were gently detached and resuspended (2×10^6 cells /ml) in PG buffer.

Platelets for AA metabolite studies, subcellular fractionation and Western blot analysis were isolated from supernatants (platelet rich plasma, PRP) after centrifugation of leukocyte concentrates on LSM 1077 cushions. PRP was mixed with PBS buffer (pH 5.9, 3:2, v/v) and centrifuged (2,000 × g, 10 min, RT), the pellet was resuspended in PBS (pH 5.9) / 0.9% NaCl (1:1, v/v), centrifuged again (2,000 × g, 10 min, RT) and was finally resuspended (1×10^8 cells /ml) in PG buffer.

Platelets for AA release studies were directly obtained from PRP after centrifugation of leukocyte concentrates on LSM 1077 cushions and adjusted to 1×10^8 cells /ml in human plasma.

Platelets for studies on platelet aggregation were isolated from freshly drawn blood (collected in Monovettes®, Sarstedt AG & Co, Nürnbrecht, Germany, 10.6 mM trisodium citrate) from healthy donors that did not take any medication for at least 7 days. After centrifugation (240 × g, 12 min at RT), PRP was collected, transferred into tubes containing 20% (v/v) ACD buffer (85 mM trisodium citrate, 65 mM citric acid, 100 mM glucose), mixed with PBS (pH 5.9) (1:2, v/v), centrifuged at 1,240 × g (7 min at RT) and the pellet was resuspended (2×10^8 /ml) in Tyrode's buffer (129 mM NaCl, 8.9 mM $NaHCO_3$, 0.8 mM KH_2PO_4, 0.87 mM $MgCl_2$, 5.6 mM glucose, 10 mM HEPES pH 7.4).

Cell counting

Cell counts were determined by trypan blue exclusion. The cell suspension was mixed in equal parts with trypan blue solution (0.2%, w/v) and cells were counted on a Bürker haemocytometer under a light microscope.

Cell viability

To exclude toxic effects of test compounds during incubation periods, PMNL and monocyte viability was analysed by light microscopy and trypan blue exclusion. Incubation with 30 µM of the isolated test compounds or 30 µg/ml of the extracts for 30 min at 37 °C caused no significant change in neutrophil or monocyte viability.

3.6 Animals

Male adult Wistar Han rats (200-230 g, Harlan, Milan, Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care complied with Italian regulations on protection of animals used for experimental and other scientific purpose (Ministerial Decree 116192) as well as with the European Economic Community regulations (Official Journal of E.C. L 358/1 12/18/1986).

3.7 Induction of mPGES-1 in A549 cells and isolation of microsomes

A549 cells (2×10^6 cells in 20 ml DMEM/high glucose (4.5 g/l) medium containing FCS (10%, v/v), penicillin (100 U/ml) and streptomycin (100 µg/ml)) were plated in flasks and incubated for 16 h at 37 °C and 6% CO_2. Subsequently, the medium was replaced by fresh medium containing 2% (v/v) of FCS. Induction of mPGES-1 expression was started by addition of interleukin-1β (IL-1β, 2 ng/ml) for 72 h (37 °C, 6% CO_2). The cells were detached, washed in PBS and the pelleted cells were frozen in liquid nitrogen. Ice cold homogenization buffer (0.1 M potassium phosphate buffer pH 7.4, 1 mM phenylmethylsulphonylfluoride (PMSF), 60 µg/mL soybean trypsin inhibitor (STI), 1 µg/mL leupeptin, 2.5 mM glutathione and 250 mM sucrose) was added and after 15 min, the pellet was resuspended and sonicated on ice (3 × 20 sec). The homogenate was subjected to differential centrifugation at 10,000 × g for 10 min and at 174,000 × g for 1 h at 4 °C. The pellet (microsomal fraction) was resuspended in 1 ml homogenization buffer and the protein concentration was determined by Bradford protein quantification [568].

3.8 Determination of PGE_2 synthase activity in microsomes of A549 cells

Microsomal membranes of A549 cells were diluted to 100 µl in homogenization buffer (see chapter 3.7) and test compounds or vehicle (DMSO) were added. After 15 min at 4 °C, PGE_2 formation was initiated by addition of PGH_2 (20 µM). After 1 min at 4 °C, the reaction was terminated by addition of 100 µl stop solution (40 mM $FeCl_2$, 80 mM citric acid and 10 µM of 11β-PGE_2). PGE_2 was separated by solid phase extraction on RP-C18 material using acetonitrile (200 µl) as eluent and analyzed by RP-HPLC (30% acetonitrile, 70% water and 0.007% trifluoroacetic acid (v/v), Nova-Pak® C18 column, 5 × 100 mm, 4 µm particle size, flow rate 1 ml/min) with UV detection at 198 nm. 11β-PGE_2 was used as internal standard to quantify PGE_2 product formation by integration of the area under the peaks.

3.9 Recombinant production of His-tagged cPLA$_2$α in Sf9 cells

The baculovirus, carrying the genetic information of the His-tagged cPLA$_2$α protein was kindly provided by Dr. M. Hoffmann (University of Frankfurt, Germany) [569]. In brief, the sequence coding for the cPLA$_2$α protein was cloned from pVL1393 into the pFastBac^{TM} HT A vector containing a 6 × His-tag coding sequence. The vector was transformed and amplified in DH10Bac^{TM} *E. coli* and Sf9 insect cells were transfected with the purified recombinant bacmid DNA via Cellfectin® reagent. The generated baculovirus was amplified by further infections.
For expression of His-tagged cPLA$_2$α, Sf9 cells were infected with recombinant baculovirus. Cells were harvested 72 h after infection, resuspended in lysis buffer (50 mM NaH_2PO_4 (pH 8), 300 mM NaCl, 10% glycerol (v/v), 1 mM EDTA, 60 µg/ml STI, 1 µg/ml leupeptin, 300 nM okadaic acid) and lysed by sonification (Branson Sonifier cell disruptor B15, Danbury, CT, USA). Insoluble particles in the cell lysate were removed by centrifugation at 100,000 × g (70 min, 4 °C). After addition of 2 mM $MgSO_4$ and 10 mM imidazole, the supernatant was incubated with Ni-NTA agarose beads for 1 h at 4 °C under continuous agitation. Beads were washed six times with wash buffer (50 mM NaH_2PO_4 buffer (pH 8), 300 mM NaCl, 10% glycerol (v/v)) containing 20 mM imidazole, and His-tagged cPLA$_2$α was eluted with wash buffer containing 150 mM imidazole. The eluate was dialyzed over night against TGN buffer (10 mM Tris-HCl (pH 8), 300 mM NaCl, 20% glycerol (v/v)) and the protein content was analyzed using a protein quantification test kit according to the manufacturer's instructions (Roti® Nanoquant, Carl Roth GmbH & Co. KG, Karlsruhe, Germany).

3.10 Determination of arachidonic acid release from artificial vesicles

The cPLA$_2$α activity assay was performed with large unilamellar vesicles (LUVs) containing PAPC (166.7 nmol per ml dispersion) and POG (83.3 nmol per ml dispersion). Alternatively, LUVs containing different ratios of PAPC and POG, PAPE or combinations of PAPC and cholesterol were used, always maintaining an overall lipid content of 250 nmol per ml dispersion. LUVs were produced by drying phospholipid solutions (in chloroform) under argon atmosphere. Vesicle buffer (134 mM NaCl, 20 mM Tris-HCl pH 7.4, with or without 1 mg/ml FAF-BSA was added and the lipid suspension was subjected to freeze-thaw cycles (liquid nitrogen / 37 °C). Lipid aggregates were disintegrated to LUVs by extrusion (100 nm pore diameter). LUVs (0.2 ml dispersion) were supplemented with EDTA 1 mM or $CaCl_2$ 1 mM and pre-incubated with test compounds 10 min (RT) prior to starting the reaction with 2.5 µg/ml of purified enzyme. After 1 h

at 37 °C, the reaction was stopped by addition of 1.6 ml methanol. 1 nmol of linolenic acid was added, samples were acidified with 40 µL HCl (1 M) and buffered with 1.6 ml PBS buffer. After solid phase extraction of the fatty acids (C18-columns, 100 mg, UCT, Bristol, PA, USA) the fatty acids were derivatized with p-anisidinium chloride using EDC and then analyzed by HPLC (Nova-Pak® C18 column (5 × 100 mm, 4 µm particle size, Waters, Eschborn, Germany)) at 250 nm using gradient elution starting from aqueous methanol 85% plus 0.007% trifluoro acetic acid (v/v) to methanol 100% plus 0.007% trifluoroacetic acid (v/v). The amount of released and derivatized AA was determined by peak area integration using derivatized linolenic acid as internal standard.

3.11 Expression and purification of human recombinant 5-LO from *E. coli*

E. coli BL21 cells were transformed with pT3-5LO plasmid as described [570]. Cells were grown overnight at 37 °C in Luria-Bertani (LB) medium containing 100 µg/ml ampicillin. LB medium containing 10 µM $FeSO_4$, 2 mM $MgSO_4$ and 100 µg/ml ampicillin was inoculated with overnight culture (30 °C) and 5-LO expression was induced by 800 µM isopropyl-β-D-thiogalactopyranoside (IPTG), when the optical density (OD, 620 nm) exceeded 0.2. At an OD (620 nm) of 2.0 (ca. 16 h after addition of IPTG), the cells were harvested (7,700 × g, 15 min, 4 °C), lysed by incubation in lysis buffer (50 mM triethanolamine/HCl (pH 8.0), 5 mM EDTA, 60 µg/ml STI, 1 mM PMSF, 2 mM dithiothreitol (DTT) and 500 µg/ml lysozyme) and homogenized by sonification (3 × 15 s) (Branson Sonifier cell disruptor B15, Danbury, CT, USA) on ice. The homogenate was centrifuged (40,000 × g, 20 min, 4 °C, Sorvall RC 5B plus, Thermo Fisher Scientific Inc., Newtown, CT, USA) and the supernatant was subjected to affinity chromatography on an ATP agarose column [362]. Elution was conducted with 20 mM ATP in PBS plus 1 mM EDTA and the eluate was used for activity assays on purified 5-LO.

3.12 Determination of 5-LO product formation in purified recombinant 5-LO

0.5 µg of purified recombinant 5-LO were diluted in 1 ml of cold PBS containing 1 mM EDTA and 1 mM ATP. Test compounds were added and after 10 min at 4 °C the samples were set to 37 °C for 30 sec. The reaction was started by addition of 2 mM $CaCl_2$ and 20 µM AA and stopped after 10 min by addition of 1 ml cold methanol. After addition of 200 ng PGB_1, acidification with 30 µl 1 M HCl and dilution with 500 µl PBS buffer, PGB_1 and the 5-LO products (5(S)-HETE and the all-trans isomers of LTB_4) were extracted via solid phase extraction on C18-columns (100 mg, UCT, Bristol, PA, USA). The extracted samples were analyzed via HPLC on a Nova-Pak® C18

column (5 × 100 mm, 4 µm particle size, Waters, Eschborn, Germany) using aqueous methanol 76% plus 0.007% trifluoroacetic acid (v/v) as mobile phase. The amount of 5-LO product formation was determined by peak area integration of the chromatograms at 280 nm (PGB_1 and the all-trans isomers of LTB_4) and 235 nm (5-HETE), using PGB_1 as internal standard [571].

3.13 Determination of the activity of isolated COX-1 and -2

Determination of the activity of isolated ovine COX-1 and human recombinant COX-2 was performed as described [572-573]. In brief, purified COX-1 (ovine, 50 units) or COX-2 (human recombinant, 20 units) were diluted in 1 ml of Tris buffer (100 mM, pH 8) supplemented with 5 mM glutathione, 5 µM haemoglobin and 100 µM EDTA at 4 °C and incubated with the test compounds for 9 min. Samples were warmed up to 37 °C for 1 min and AA (5 µM for COX-1, 2 µM for COX-2) was added to start the reaction. After 5 min at 37 °C, the reaction was stopped by addition of ice-cold methanol. After addition of 200 ng PGB_1, acidification with HCl (30 µl 1 M) and dilution with PBS buffer (500 µl), COX-1 product 12-HHT was extracted via solid phase extraction on C18-columns (100 mg, UCT, Bristol, PA, USA). The extracted samples were analyzed via HPLC on a Nova-Pak® C18 column (5 × 100 mm, 4 µm particle size, Waters, Eschborn, Germany) using aqueous methanol 76% plus 0.007% trifluoroacetic acid (v/v) as mobile phase. The amount of 12-HHT was determined by peak area integration of the chromatograms at 280 nm (PGB_1) and 235 nm (12-HHT).

3.14 Determination of CG and HLE activity

Cathepsin G (CG) and human leukocyte elastase (HLE) were liberated from PMNL as described [574]. In brief, PMNL (2.5×10^7 /ml in PG buffer supplemented with 1 mM $CaCl_2$ (PGC buffer)) were warmed up to 37 °C (2 min) and stimulated with 10 µM cytochalasin B for 5 min at 37 °C and 2.5 µM fMLP for further 5 min at 37 °C. The incubation was stopped on ice and the cells were spun down (1,200 × g, 5 min, 4 °C). The supernatant was used as source for CG and HLE, which are contained in concentrations of about 10 µg/ml each [574]. The protein content in the supernatant was determined by Bradford protein quantification [568] and adjusted to 150 µg/ml with PGC buffer, which corresponds to a final concentration of 7.5 µg/ml in the assay or an enzyme activity of about 0.5 mU/ml for CG and 1.5 mU/ml for HLE under the applied conditions (1 U hydrolyzes 1 µmole of substrate per minute). Alternatively, 1 µg/ml of purified human CG (Applichem GmbH, Darmstadt, Germany) were used, which corresponded to an activity of 1.4 mU per µg CG at the specified conditions.

Incubation was performed in a 96-well plate. 180 µl of assay buffer (100 mM HEPES pH 7.4, 500 mM NaCl) were mixed with 10 µl of the enzyme solution and the test compounds or vehicle (DMSO) and incubated for 10 min on ice. The reaction was started by addition of 20 µl of the respective chromogenic substrate (10 mM N-Suc-Ala-Ala-Pro-Phe-p-nitroanilide as specific substrate for CG and 1 mM N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide as specific substrate for HLE, both diluted in DMSO). The absorption of p-nitroaniline was recorded at 405 nm over a period of 60 min (CG) or 10 min (HLE) at RT in a multi-well scanning spectrophotometer (Victor^3 plate reader, Perkin Elmer LAS GmbH, Rodgau-Jügesheim, Germany). Measurements deviating from a linear increase in absorption were rejected. The increase in absorption was adjusted by the blank control (substrate in buffer without enzyme solution) and all results were related to the vehicle control.

3.15 Arachidonic acid release from isolated blood cells

Monocytes (2×10^6 /ml) and PMNL (5×10^6 /ml) in RPMI 1640 were incubated with 5 nM [^{3}H]-AA, platelets (1×10^8 /ml) were incubated with 100 µM aspirin plus 10 nM [^{3}H]-AA for 2 h at 37 °C and 6% CO_2. Monocytes and PMNL were washed twice with PG buffer containing 2 mg/ml FAF-BSA. Platelets were washed twice with PBS (pH 5.9) containing 1 mM $MgCl_2$, 11.5 mM $NaHCO_3$, 1 mg/ml glucose and 1 mg/ml FAF-BSA. Cells were resuspended in PG buffer (5×10^6 monocytes /ml, 2×10^7 PMNL /ml and 1×10^8 platelets /ml) and supplemented with 1 mM $CaCl_2$. After pre-incubation with the test compounds for 15 min at 37 °C, samples were stimulated with 1 µM A23187 for 5 min at 37 °C. The incubation was stopped on ice (10 min) and cells were spun down (monocytes and PMNL at 1,200 × g, platelets at 5,000 × g, 10 min). Aliquots (300 µl) of the supernatants were mixed with 2 ml Ultima Gold^{TM} XR and measured on a scintillation counter (Micro Beta Trilux, Perkin Elmer Inc., Waltham, MA, USA) to detect released [^{3}H]-AA.

3.16 Determination of 5-LO product and 12- and 15-HETE formation in human PMNL

For determination of 5-LO products and 12- and 15-HETE formation in intact cells, PMNL (5×10^6 in 1 ml of PG buffer) were supplemented with 1 mM $CaCl_2$ and pre-incubated with test compounds or vehicle (DMSO) for 15 min at 37 °C. After pre-incubation, the cells were stimulated at 37 °C either with 1 or 2.5 µM A23187 alone, or 2.5 µM A23187 plus 20 µM AA for 5 or 10 min. The reaction was stopped by addition of 1 ml cold methanol and 200 ng PGB_1, 30 µl 1 M HCl and 500 µl of PBS buffer were added to the samples. All samples were centrifuged at

800 × g for 10 min at RT. PGB_1 and the 5-LO products 5(S)-HETE, 5(S),12(S)-dihydroxy-6,10-trans-8,14-cis-eicosatetraenoic acid (5(S),12(S)-diHETE), LTB_4 and its all-trans isomers as well as the 12-/15-LO products 12-HETE and 15-HETE were extracted via solid phase extraction on C18-columns (100 mg, UCT, Bristol, PA, USA). The extracted samples were analyzed via HPLC on a Nova-Pak® C18 column (5 × 100 mm, 4 µm particle size, Waters, Eschborn, Germany) using aqueous methanol 76% plus 0.007% trifluoroacetic acid (v/v) as mobile phase. The amount of 5-LO products was determined by peak area integration of the chromatograms at 280 nm (PGB_1, 5(S),12(S)-diHETE, LTB_4 and its all-trans isomers) and 235 nm (5(S)-HETE, 12- and 15-HETE), using PGB_1 as internal standard [571].

3.17 Determination of 5-LO product and 12-HETE, 15-HETE and 12-HHT formation in human monocytes

For determination of 5-, 12- and 15-LO product formation in monocytes, cells (2×10^6 in 1 ml of PG buffer) were supplemented with 1 mM $CaCl_2$ and pre-incubated with test compounds or vehicle (DMSO) for 15 min at 37 °C. After pre-incubation, the cells were stimulated with 1 µM A23187 or 1 µM A23187 plus 20 µM AA for 5 min at 37 °C. The reaction was stopped by addition of 1 ml of cold methanol and 200 ng PGB_1, 30 µl 1 M HCl and 500 µl PBS buffer were added to the samples. The samples were centrifuged at 800 × g for 10 min at RT. PGB_1, the 5-LO products 5(S)-HETE, 5(S),12(S)-diHETE, LTB_4 and its all-trans isomers as well as the 15-LO product 15-HETE, the 12-LO product 12-HETE and the COX product 12-HHT were extracted via solid phase extraction on C18-columns (100 mg, UCT, Bristol, PA, USA). The extracted samples were analyzed via HPLC on a Nova-Pak® C18 column (5 × 100 mm, 4 µm particle size, Waters, Eschborn, Germany) using aqueous methanol 76% plus 0.007% trifluoroacetic acid (v/v) as mobile phase. The amount of 5-LO, 15-LO, 12-LO and COX products was determined by peak area integration of the chromatograms at 280 nm (PGB_1, 5(S),12(S)-diHETE, LTB_4 and its all-trans isomers) and 235 nm (5(S)-HETE, 15-HETE, 12-HETE and 12-HHT), using PGB_1 as internal standard.

3.18 Determination of 12-HHT and 12-HETE formation in washed human platelets

Freshly isolated platelets (10^8 in 1 ml PG buffer) were supplemented with $CaCl_2$ 1 mM and pre-incubated with the indicated substances under the conditions specified in the respective experiment. After addition of the indicated stimuli, samples were incubated for 5 min at 37 °C and

the reaction was stopped by addition of ice-cold methanol. After addition of 200 ng PGB_1, acidification with 30 µl 1 M HCl and dilution with 500 µl PBS buffer, samples were centrifuged (800 × g, 10 min, RT) and PGB_1, COX-1 product 12-HHT and 12-LO product 12-HETE were extracted via solid phase extraction on C18-columns (100 mg, UCT, Bristol, PA, USA). The extracted samples were analyzed via HPLC on a Nova-Pak® C18 column (5 × 100 mm, 4 µm particle size, Waters, Eschborn, Germany) using aqueous methanol 76% plus 0.007% trifluoroacetic acid (v/v) as mobile phase. The amount of AA metabolites was determined by peak area integration of the chromatograms at 280 nm (PGB_1) and 235 nm (12-HHT and 12-HETE).

3.19 Determination of 6-keto $PGF_{1\alpha}$ synthesis in IL-1β-primed A549 cells

A549 cells (2×10^6 cells in 20 ml DMEM/high glucose (4.5 g/l) medium containing FCS (10%, v/v), penicillin (100 U/ml) and streptomycin (100 µg/ml)) were plated in flasks and incubated for 16 h at 37 °C and 6% CO_2. Subsequently, the medium was replaced by fresh medium containing 2% (v/v) of FCS. Induction of COX-2 expression was started by addition of IL-1β (2 ng/ml) for 72 h (37 °C, 6% CO_2). After detachment, cells were washed twice with PBS buffer and resuspended (2×10^6 /ml) in PGC buffer. Cells were incubated with the respective test compounds for 10 min (37 °C) and the reaction was started by addition of 3 µM AA. After 15 min at 37 °C, the incubation was stopped in ice, the cells were spun down (800 × g, 4 °C, 5 min) and 6-keto $PGF_{1\alpha}$ in the supernatant was determined using a 6-keto $PGF_{1\alpha}$ ELISA kit (Assay designs, Ann Arbor, MI, USA) according to the manufacturer's protocol.

3.20 Determination of PGE_2 formation in LPS-primed human monocytes

Freshly isolated monocytes (10^6 cells in 1 ml RPMI 1640 medium supplemented with FCS (0.5%, v/v), penicillin (100 U/ml) and streptomycin (100 µg/ml)) were plated in 12-well plates and stimulated with LPS (1 µg/ml) for 20 h (37 °C, 6% CO_2). Subsequently, the wells were washed thrice with cold PBS buffer and fresh medium was added (1 ml per well). After 30 min at 37 °C (6% CO_2), the medium was changed again and the cells were incubated with the respective test compounds for 15 min (37 °C, 6% CO_2). The reaction was started by addition of 1 µM AA. After 30 min, the medium was collected from the wells, centrifuged (300 × g, 10 min, 4 °C) and PGE_2 formation was quantified directly in the cleared medium using a PGE_2 ELISA kit (Assay designs, Ann Arbor, MI, USA) according to the manufacturer's protocol.

3.21 Platelet aggregation

Aggregation of washed human platelets was determined using a turbidimetric light-transmittance device (two-channel aggregometer, Chrono-Log Corp., Havertown, PA, USA). Platelets in Tyrode's buffer (2×10^8 /ml) were prepared as described (see chapter 3.5) and pre-incubated with the test compounds for 15 min at 37 °C. The instrument was calibrated with unstimulated platelet suspension (0% aggregation) and the vehicle control 5 min after stimulation with the respective stimulus (100% aggregation). $CaCl_2$ (1 mM) was added to the samples just before starting of the measurement. Turbidity was recorded under continuous stirring (1,000 rpm) at 37 °C for 5 min. Data are presented in percent of light transmission in the vehicle control after total aggregation.

3.22 Sample preparation for Western blot analysis from platelets

Washed human platelets (10^9 /ml) were resuspended in PG buffer and either pre-treated with 1 mM EDTA and 30 µM BAPTA-AM (30 min prior to incubation, RT) or 1 mM $CaCl_2$ (5 min prior to incubation). The samples were warmed to 37 °C 4 min prior to incubation and stimulated with the test compounds or vehicle (DMSO). The incubation was stopped by addition (1:1, v/v) of SDS loading buffer (2 ×) (20 mM Tris-HCl pH 8, 2 mM EDTA, 5% SDS (w/v), 10% β-mercaptoethanol (v/v)) and the samples were heated for 5 min at 95 °C. After addition (20%, v/v) of bromophenol blue (0.05%, w/v in aqueous glycerol (50%, v/v)), the samples were subjected to SDS-PAGE and Western blot analysis as described (see chapters 3.29 and 3.30).

3.23 Subcellular fractionation of washed human platelets

Washed human platelets (5×10^9 /ml) were resuspended in translocation buffer (134 mM NaCl, 15 mM Tris-HCl pH 7.6, 1 g/l glucose) and either pre-treated with 1 mM EDTA and 30 µM BAPTA-AM (30 min prior to incubation, RT) or 1 mM $CaCl_2$ (5 min prior to incubation). Then, the temperature was increased to 37 °C (within 4 min) and the test compounds or vehicle (DMSO) were added. After 5 min, the incubation was stopped on ice. Protease inhibitors (10 µg/ml leupeptin, 60 µg/ml STI and 1 mM PMSF) were added and the cells were lysed by three freeze-thaw cycles (liquid nitrogen/water bath at RT) and centrifuged at 100,000 × g (45 min, 4 °C). The supernatant (cytosolic fraction) was collected and the pellet (membranous fraction) was resuspended in translocation buffer with protease inhibitors. Both fractions were mixed (1:1, v/v) with SDS loading buffer (2 ×) (20 mM Tris-HCl pH 8, 2 mM EDTA, 5% SDS (w/v), 10% β-

mercaptoethanol (v/v)) and heated for 5 min at 95 °C. A solution of bromophenol blue (0.05%, w/v) in aqueous glycerol (50%, v/v) was added (20%, v/v) and the samples were analyzed by Western blotting on cPLA$_2$α.

3.24 Measurement of intracellular Ca^{2+} levels

Washed platelets (6×10^8 /ml) in PG buffer were incubated with 2 µM Fura-2-AM for 30 min at 37 °C under light protection to avoid photobleaching. After washing out excessive dye with PG buffer, cells were resuspended (10^8 /ml) in PG buffer and incubated with the test compounds for 15 min at RT. Subsequently, samples were transferred to a thermally controlled and constantly stirred fluorometer cuvette (37 °C) in a fluorospectrometer (Aminco-Bowman series 2, Thermo Fisher Scientific Inc., Rochester, NY, USA). $CaCl_2$ (1 mM) was added 1 min prior to stimulation with the indicated stimuli. Fluorescence emission at 510 nm was measured after excitation at 340 nm and 380 nm and intracellular Ca^{2+} was calculated according to Grynkiewicz et al. [575]. Maximal fluorescence was determined by lysing cells with 0.5% (v/v) Triton-X 100 and minimal fluorescence after chelation with 10 mM EDTA.

3.25 Dual-luciferase glucocorticoid receptor response element reporter assay

A549 cells (2×10^4 cells in 100 µl RPMI 1640 medium supplemented with 2% FCS) were transfected with a mixture of an inducible glucocorticoid receptor responsive firefly luciferase reporter construct and a constitutively expressing *Renilla* luciferase construct (100 ng) using the Cignal^{TM} GRE Reporter Assay Kit (SABiosciences corp., Frederick, MD, USA) and the transfection reagent SureFECT^{TM} (SABiosciences corp., Frederick, MD, USA) according to the manufacturer's instructions. The constitutively expressing *Renilla* luciferase construct serves as an internal control for normalizing transfection efficiency and monitoring cell viability. A negative control composed of a non-inducible firefly luciferase reporter construct and a constitutively expressing *Renilla* luciferase construct (100 ng) was carried out for each sample to exclude unspecific effects or spontaneous reporter activity. After 24 h (37 °C, 6% CO_2), the medium was changed to RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin and the cells were allowed to recover for 16 h. Successful transfection was checked by fluorescence microscopy, monitoring the positive control, which constitutively expressed green fluorescent protein (GFP) along with firefly and *Renilla* luciferases. Then, the cells were treated with the test substances or vehicle (DMSO) for 6 h (37 °C, 6% CO_2) and the incubation was

stopped by cell lysis. For passive cell lysis and differential determination of the luciferases' activities, a dual-luciferase reporter assay system (Promega GmbH, Mannheim, Germany) was used according to the manufacturer's instructions. Luciferase activity was measured in a luminometer (Victor^3 plate reader, Perkin Elmer LAS GmbH, Rodgau-Jügesheim, Germany).

3.26 Determination of PGE_2 and 6-keto $PGF_{1\alpha}$ formation in LPS-stimulated whole blood

Whole blood, freshly withdrawn by venipuncture from healthy adult donors, was obtained from the University Hospital of Tübingen. Blood was collected in Monovettes® (Sarstedt AG & Co, Nürnbrecht, Germany) containing 10-30 I.U. heparin/ml. Aliquots of 0.8 ml whole blood were treated with thromboxane synthase inhibitor CV-4151 (1 µM) and buffered with 0.2 ml potassium phosphate buffer (10 mM potassium phosphate buffer pH 7.4, 3 mM KCl, 140 mM NaCl and 6 mM glucose). After pre-incubation with substances or vehicle (DMSO) for 10 min at RT, the samples were stimulated with LPS (10 µg/ml) for 5 h at 37 °C. The reaction was stopped on ice and the samples were centrifuged (2,300 × g, 10 min, 4 °C). 6-keto $PGF_{1\alpha}$ formation was quantified directly in the plasma using a 6-keto $PGF_{1\alpha}$ ELISA kit (Assay designs, Ann Arbor, MI, USA) according to the manufacturer's protocol. For quantification of PGE_2 formation, the supernatant was acidified with citric acid (30 µL, 2 M) and the plasma was centrifuged again (2,300 × g, 10 min, 4 °C). Solid phase extraction of the supernatant and HPLC analysis of PGE_2 was performed as described above (see chapter 3.8). The PGE_2 peak, identified by co-elution with the authentic standard, was collected and acetonitrile was removed under a nitrogen stream. The pH was adjusted to 7.4 by addition of 10 × PBS buffer before PGE_2 contents were quantified using a PGE_2 ELISA kit (Assay designs, Ann Arbor, MI, USA) according to the manufacturer's protocol.

3.27 Determination of 5-LO product, 12-HETE and 12-HHT formation in human whole blood

Whole blood, freshly withdrawn by venipuncture from healthy adult donors, was obtained from the University Hospital of Tübingen. Blood was collected in Monovettes® (Sarstedt AG & Co, Nürnbrecht, Germany) containing 10-30 I.U. heparin/ml. After pre-incubation of aliquots of 2 ml with the test compounds or vehicle (DMSO) for 10 min at 37 °C, samples were stimulated with A23187 (30 µM). After 10 min, the incubation was stopped on ice and the samples were centrifuged (600 × g, 10 min, 4 °C). 500 µl of the supernatant were mixed with 2 ml of methanol

and 200 ng PGB_1. The samples were stored at -20 °C for 2 h and centrifuged again (600 × g, 10 min, 4 °C) to spin down precipitated protein. The supernatants were mixed with 2.5 ml PBS buffer and 75 µl 1 M HCl. PGB_1 and the 5-LO products 5(S)-HETE, 5(S),12(S)-diHETE and LTB_4 and its all-trans isomers as well as the 12-LO product 12-HETE and the COX product 12-HHT were extracted via solid phase extraction on C18-columns (100 mg, UCT, Bristol, PA, USA). The extracted samples were analyzed via HPLC on a Nova-Pak® C18 column (5 × 100 mm, 4 µm particle size, Waters, Eschborn, Germany) using aqueous methanol 76% plus 0.007% trifluoroacetic acid (v/v) as mobile phase. The amount of 5-LO, 12-LO and COX products was determined by peak area integration of the chromatograms at 280 nm (PGB_1, 5(S),12(S)-diHETE and LTB_4 and its all-trans isomers) and 235 nm (5(S)-HETE, 12-HETE and 12-HHT) using PGB_1 as internal standard.

3.28 Carrageenan-induced pleurisy in rats

Test compounds were dissolved in DMSO, diluted in saline (1:25, v/v) and administered intraperitoneally (i.p.) in an overall volume of 1.5 ml 30 min before administration of carrageenan. The vehicle-treated group of rats received 1.5 ml of DMSO 4% in saline. Rats were anaesthetized with enflurane 4% (v/v) in a mixture of O_2, 0.5 l/min and N_2O, 0.5 l/min, and submitted to a skin incision at the level of the left sixth intercostal space. The underlying muscle was dissected, and saline (0.2 ml) or λ-Carrageenan type IV 1% (w/v) (0.2 ml) was injected into the pleural cavity. The incision was closed with wound clips and the animals were allowed to recover. At 4 h after the injection of λ-carrageenan, the animals were killed by inhalation of CO_2. After opening of the abdominal wall, the pleural cavity was punctured and 2 ml of saline solution containing heparin (5 U/ml) were injected in order to wash the pleural cavity. The exudate and washing solution were removed by aspiration, and the total volume was measured using adjustable volume pipettes. Any exudate that was contaminated with blood was discarded. The amount of exudate was calculated by subtracting the injected volume (2 ml) from the total recovered volume. Leukocytes in the exudate were spun down (800 × g, 10 min) and resuspended in PBS for cell counting (see chapter 3.5). The amounts of PGE_2, LTB_4 and 6-keto $PGF_{1\alpha}$ in the supernatant of centrifuged exudate were assayed by radioimmunoassay (PGE_2, Sigma-Aldrich S. r. l., Milan, Italy) and ELISA (LTB_4 and 6-keto $PGF_{1\alpha}$, Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's protocol. The results of the eicosanoids are expressed in pg per rat, the exudate volume is expressed in µl per rat and the cell count in million per rat and represent the mean ± S.E. of 5 - 20 rats.

3.29 Sodium dodecylsulphate polyacrylamide gel electrophoresis

Samples were analyzed per sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) after boiling in the same volume of 2 × SDS loading buffer (20 mM Tris-HCl pH 8, 2 mM EDTA, 5% SDS (w/v), 10% β-mercaptoethanol (v/v)) for 5 min at 95 °C, sonification and supplementation (20%, v/v) with bromophenol blue (0.05%, w/v in aqueous glycerol (50%, v/v)). 10 µl of sample were loaded on polyacrylamide gels (8-12% polyacrylamide, depending on the molecular weight of the analyzed proteins) and discontinuous electrophoresis was performed using a Mini-PROTEAN system (Bio-Rad Laboratories Inc., Hercules, CA, USA) as described [576]. The molecular weight of the proteins was estimated by comparison with pre-stained broad range molecular weight marker peqGold Protein Marker IV (peqLab Biotechnology GmbH, Erlangen, Germany).

3.30 Western blot analysis

The gels from the SDS-PAGE were blotted to nitrocellulose membranes (Hybond™ ECL membrane, GE healthcare, Munich, Germany) by the tank blotting method (Bio-Rad Mini Trans-Blot® cell, Bio-Rad Powerpac™ Basic, Bio-Rad Laboratories Inc., Hercules, CA, USA) in transfer buffer (48 mM Tris-HCl, 40 mM glycine, 0.1 mM SDS, 20% methanol (v/v)). After electroblot, uniform protein loading was confirmed by staining with Ponceau S (5% (w/v) in 5% (v/v) acetic acid). The membranes were blocked with 5% BSA (w/v) in TBS-T buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 0.1% Tween 20 (v/v)) for 1 h at RT. After washing in TBS-T, the membranes were incubated in the respective primary antibodies overnight at 4 °C (diluted in blocking buffer plus 0.05% NaN_3 (w/v), Table **4**). The membranes were washed in TBS-T and incubated in secondary antibody for 1-3 h at RT (diluted 1:1,000 (v/v) in TBS-T plus 5% FCS (v/v) for alkaline phosphatase-coupled antibodies, 1:2,500 (v/v) in TBS-T for fluorescent dye-coupled antibodies, 1:10,000 (v/v) in TBS-T for peroxidase-coupled antibodies).

After washing, alkaline phosphatase-coupled antibodies were visualized with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (0.4 mM each) in detection buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) and scanned on a flat bed scanner (AGFA SnapScan 1236, AGFA Graphics Germany GmbH & Co. KG, Düsseldorf, Germany).

Fluorescent dye-coupled antibodies (Cy3- and Cy5-coupled) were dried after washing and scanned on a fluorescence scanner (Ettan™DIGE system, GE Healthcare Europe GmbH, Munich, Germany).

Peroxidase-coupled antibodies were treated with chemoluminescence substrate (Lumi-Light or Lumi-Light^{Plus}, Roche Diagnostics GmbH, Mannheim, Germany), the membranes were exposed to an autoradiography film (Amersham Hyperfilm ECL, GE Healthcare GmbH, Munich, Germany) for 10 sec to 30 min and the film was developed (CP 1000, AGFA Healthcare N.V., Mortsel, Belgium).

Table 4: Primary antibodies.

Antigen of primary antibody	Source	Dilution (v/v)	Provider
Phospho-Akt (Ser^{473})	Rabbit, polyclonal antibody	1:1,000	Cell Signaling Technology Inc., Danvers, MA, USA
β-actin	Rabbit, monoclonal antibody	1:1,000	Cell Signaling Technology Inc., Danvers, MA, USA
$cPLA_2$	Rabbit, polyclonal antibody	1:200	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
Phospho-$cPLA_2$ (Ser^{505})	Rabbit, polyclonal antibody	1:1,000	Cell Signaling Technology Inc., Danvers, MA, USA
Phospho-JNK (p.-Jun-amino-terminal kinase) (Thr^{183}/Tyr^{185})	Mouse, monoclonal antibody	1:1,000	Cell Signaling Technology Inc., Danvers, MA, USA
Phospho-p42/44 MAPK (p.-ERK-1/-2) (Thr^{202}/Tyr^{204})	Mouse, monoclonal antibody	1:1,000	Cell Signaling Technology Inc., Danvers, MA, USA
Phospho-p38 MAPK (Thr^{180}/Tyr^{182})	Rabbit, polyclonal antibody	1:1,000	Cell Signaling Technology Inc., Danvers, MA, USA

3.31 Statistical analysis

Data are expressed as mean + standard error (S.E.) of a certain number of independent observations (n), unless stated otherwise. Statistical evaluation of data was performed by one-way analysis of variance (ANOVA) for independent or correlated samples followed by Tukey HSD or Tukey-Kramer *post-hoc* tests (GraphPad InStat, GraphPad Software Inc., La Jolla, CA, USA). A P-value of < 0.05 (*), < 0.01 (**) or < 0.001 (***) was considered significant.

IC_{50} values were determined by fitting concentration response data to a four parameter logistic curve (SigmaPlot, Systat Software Inc., San Jose, CA, USA) or by graphic linear interpolation.

4 Results

4.1 Effect of lupeolic acids on cPLA$_2$α activity

BAs have been shown to interfere with several target structures in AA metabolism (see chapter 2.5.4.3). This chapter highlights direct effects of triterpenic acids from *Boswellia* spec. on cPLA$_2$α-driven AA release.

4.1.1 Effects of triterpenic acids on cPLA$_2$α activity in a cell-free assay

We first investigated the effect of triterpenic acids isolated from the resin of *Boswellia* species on isolated cPLA$_2$α. Therefore, the human cPLA$_2$α protein was recombinantly expressed in Sf9 cells and purified by affinity chromatography and dialysis. Artificial mixed vesicles composed of defined phospholipids were used as substrate. Palmitoyl-oleoyl-glycerol was added to the phosphatidylcholine lipids to alleviate the accessibility of the substrate and thereby enhance cPLA$_2$α activity [211]. Incubation of recombinant cPLA$_2$α with these vesicles led to marked mobilization of AA that was inhibited after withdrawal of Ca^{2+} by EDTA or by the well-recognized synthetic cPLA$_2$α control inhibitor RSC-3388 of the pyrrolidine family of cPLA$_2$α inhibitors ("cPLA$_2$α-i.") [235]. In this test system, most of the triterpenic acids were only hardly effective at a concentration of 10 µM (Figure **10**). The only compound showing discernable, concentration-dependent activity was Ac-OH-LA, an LA derivative that is hydroxylated in the C-28 position. Its impact on cPLA$_2$α activity was even more obvious when BSA was omitted from the assay. Here, a divergent pattern for the LAs lacking the 28-OH group and the C-28-hydroxylated analogue became evident (Figure **11**). LA and Ac-LA only exhibited a maximal inhibition of 44.8% and 29.7% (at 30 µM), respectively, whereas Ac-OH-LA inhibited AA release comparably to the cPLA$_2$α control inhibitor (82% inhibition) with an IC_{50} value of 3.0 µM. These data indicate that Ac-OH-LA is a direct inhibitor of cPLA$_2$α.

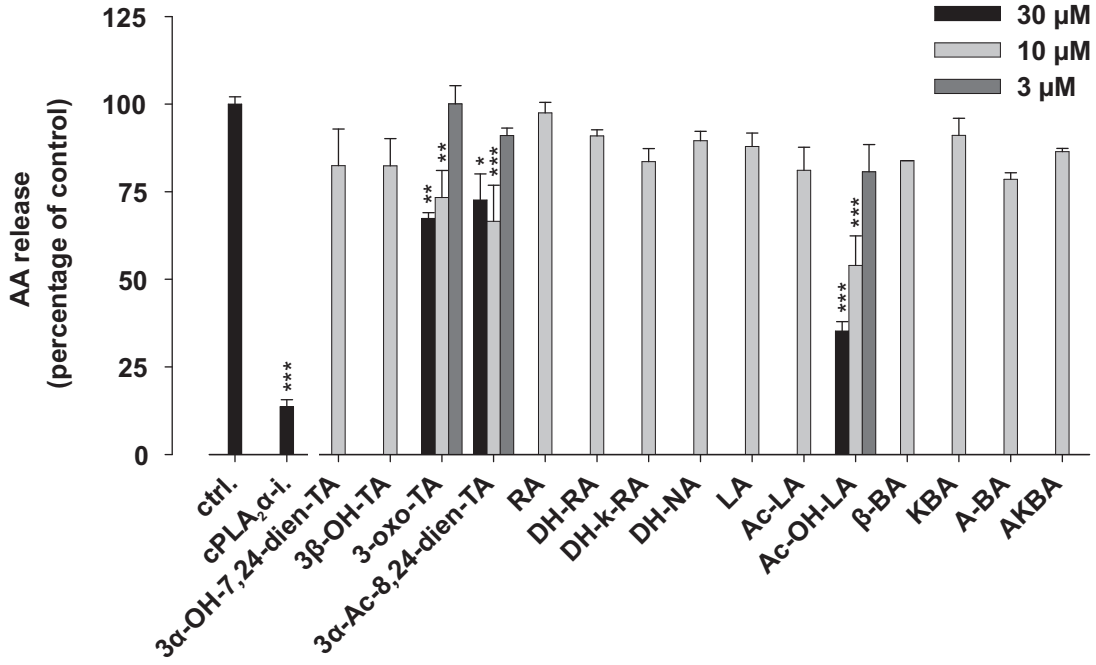


Figure 10: Effects of triterpenic acids from *Boswellia* species on AA release from PAPC/POG vesicles by purified cPLA$_2$α. PAPC/POG-vesicles (lipid concentration 250 µM in TBS buffer containing 1 mg/ml FAF-BSA) were supplemented with $CaCl_2$ (1 mM) and pre-incubated with vehicle (ctrl., DMSO), cPLA$_2$α inhibitor (cPLA$_2$α-i.) 5 µM or triterpenic acids at the indicated concentrations for 10 min at RT. The reaction was started by addition of the purified cPLA$_2$α enzyme (2.5 µg/ml) and maintained at 37 °C for 60 min. After derivatization, AA was analyzed by HPLC. Data are given as mean + S.E. of the percentage of the vehicle control (=100%), n = 3 – 6, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey-Kramer *post hoc* test.

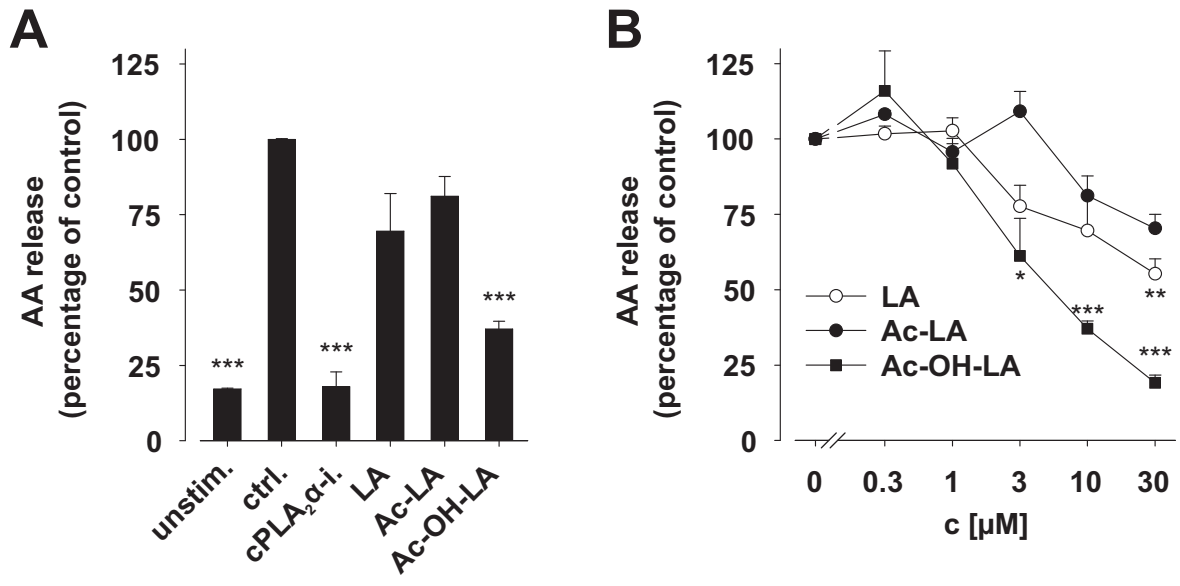


Figure 11: Effects of lupeolic acids on AA release from PAPC/POG vesicles by purified cPLA$_2$α. PAPC/POG-vesicles (lipid concentration 250 µM in TBS buffer without BSA) were supplemented with $CaCl_2$ (1 mM) and pre-incubated with vehicle (ctrl., DMSO), 5 µM cPLA$_2$α inhibitor (cPLA$_2$α-i.) and LA, Ac-LA and Ac-OH-LA (10 µM in panel **A**, and at the indicated concentrations in panel **B**) for 10 min at RT. The reaction was started by addition of the purified cPLA$_2$α enzyme (2.5 µg/ml) and maintained at 37 °C for 60 min. After derivatization, AA was analyzed by HPLC. Data are given as mean + S.E. of the percentage of the vehicle control, n = 3 – 5, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey-Kramer *post hoc* test.

4.1.2 Effects of lupeolic acids on arachidonic acid release in different isolated human blood cells

To pursue the inhibitory effect of LAs in a cellular environment, human blood cells were isolated, labelled with [^{3}H]-AA, pre-incubated with LAs and stimulated with calcium ionophore A23187. A23187 leads to a massive influx of extracellular Ca^{2+} into the cell, which provokes translocation of the cPLA$_2$α to its substrate in cellular membranes. In platelets, stimulation with A23187 (1 µM) enhanced AA release about threefold which was essentially prevented by pre-incubation with the cPLA$_2$α control inhibitor (Figure **12 A**). Again, LAs lacking the 28-OH group were less effective in inhibiting A23187-induced AA release (maximal inhibition of 50.1% and 37.7% for LA and Ac-LA, respectively at 10 µM), which was not enhanced by increasing concentrations (Figure **12 A** and **B**). On the other hand, Ac-OH-LA showed a concentration-dependent inhibition of stimulus-induced AA release with almost complete blocking at 30 µM and an IC_{50} value of 1.9 µM. Also in A23187-stimulated monocytes (Figure **12 C** and **D**), LAs lacking the 28-OH group did not affect AA release at concentrations up to 30 µM. However, at a concentration of 10 µM, Ac-OH-LA inhibited AA release to the level of the unstimulated control or the cPLA$_2$α control inhibitor (IC_{50} value of 4.7 µM). The same pattern of effects was observed in A23187-induced AA release in PMNL (Figure **12 E**). Here, stimulation with A23187 (1 µM) even led to sevenfold increase in the release of AA that was vastly inhibited by the cPLA$_2$α control inhibitor again. At 10 µM, LAs lacking the 28-OH group did not affect AA release at all, but Ac-OH-LA showed inhibition of 87.5%. Together, A23187-induced AA release in various blood cells is prevented by treatment with Ac-OH-LA, whereas LAs lacking the 28-OH group are rather ineffective.

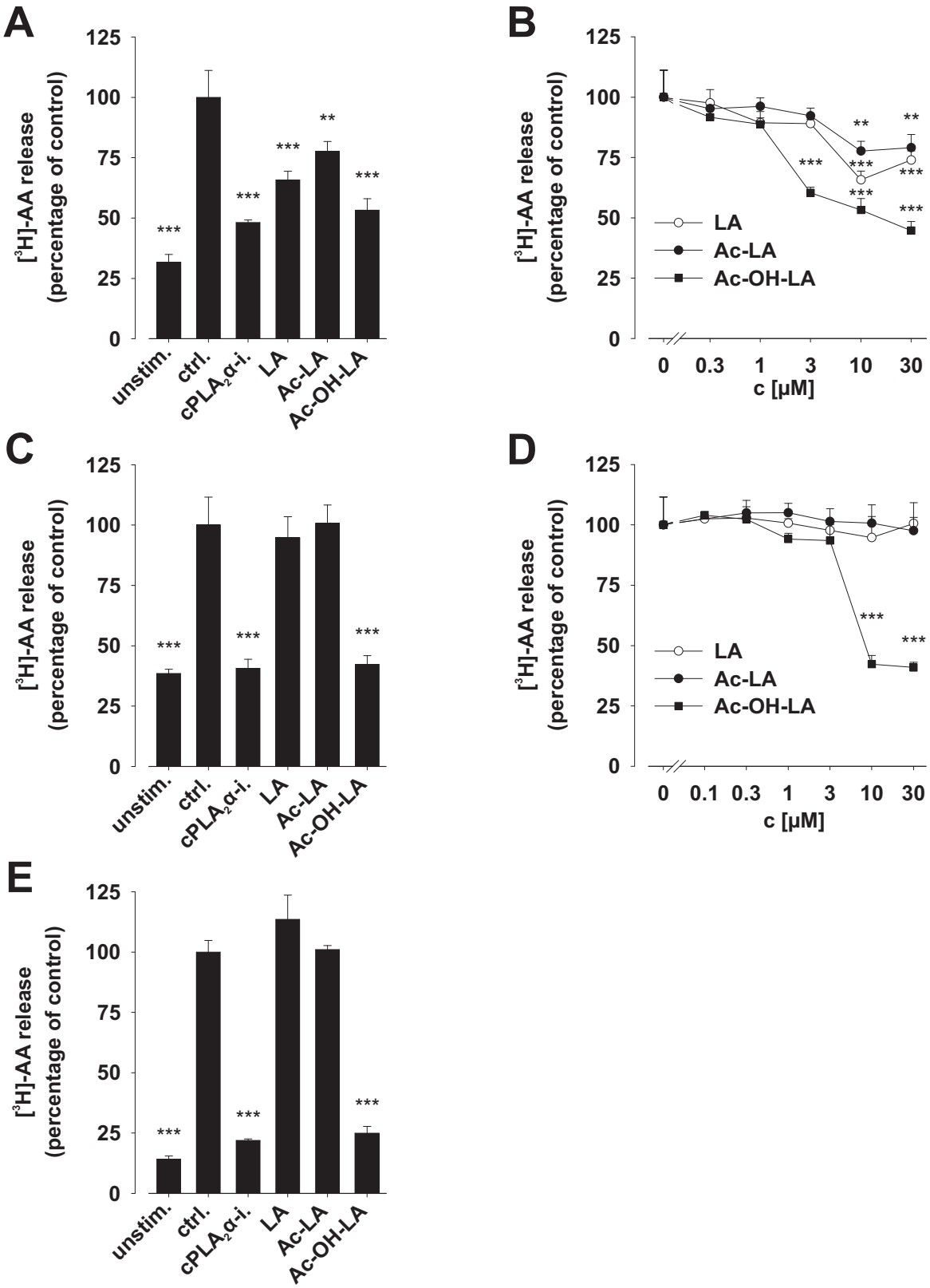


Figure 12: Effects of LAs on AA release from isolated human blood cells. [^{3}H]-AA-labelled platelets (**A** and **B**, 10^8 /ml in PGC buffer), monocytes (**C** and **D**, 5×10^6 /ml in PGC buffer), or PMNL (**E**, 2×10^7 /ml in PGC buffer) were pre-incubated with vehicle (ctrl., DMSO), 5 µM cPLA$_2$α inhibitor (cPLA$_2$α-i.), LA, Ac-LA and Ac-OH-LA (10 µM each in **A**, **C** and **E** and at the indicated concentrations in **B** and **D**) for 15 min at 37 °C. The cells were stimulated with 1 µM A23187 for 5 min at 37 °C. The reaction was terminated on ice, the samples were centrifuged and the supernatant was analyzed for [3H]-AA (and its derivatives) by scintillation counting. Data are given as mean + S.E. of the percentage of the vehicle control (+1 µM A23187) with $(13.5 \pm 1.50) \times 10^3$ cpm (**A** and **B**), $(29.7 \pm 3.42) \times 10^3$ cpm (**C** and **D**) and $(23.7 \pm 1.14) \times 10^3$ cpm (**E**), n = 3 – 5, ** $p < 0.01$, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey-Kramer *post hoc* test.

4.1.3 Impact of lupeolic acids on arachidonic acid metabolite formation in human blood cells

Because AA serves as substrate for the synthesis of several eicosanoids (e.g. PGs and LTs) we aimed to investigate whether Ac-OH-LA, which suppresses AA release, might also reduce the release of eicosanoids from the respective human blood cells. Stimulation of platelets with A23187 led to a marked production of the COX-1 product 12-HHT and the 12-LO product 12-HETE, which was prevented by inhibition of cPLA$_2$α (using cPLA$_2$α-i.) or by inhibition of COX-1 or of 12-LO by ibuprofen or CDC, respectively. Ac-OH-LA performed concentration-dependent inhibition of both 12-HHT (Figure **13 A** and **B**) and 12-HETE formation (Figure **13 C** and **D**). At 10 µM, Ac-OH-LA blocked 12-HHT production to an extent that was comparable to the COX inhibitor ibuprofen (30 µM). Also blocking of 12-HETE formation by Ac-OH-LA (10 µM) was comparable to the effect of the 12-LO inhibitor CDC (10 µM). For both AA metabolites the IC_{50} values were in the same range with 3.0 µM (12-HHT) vs. 3.5 µM (12-HETE). On the other hand, LAs lacking the 28-OH group did not show distinct effects on AA metabolite formation. Interestingly, the cPLA$_2$α control inhibitor only partially inhibited 12-HHT production at a concentration of 15 µM, but almost totally blocked 12-HETE production.

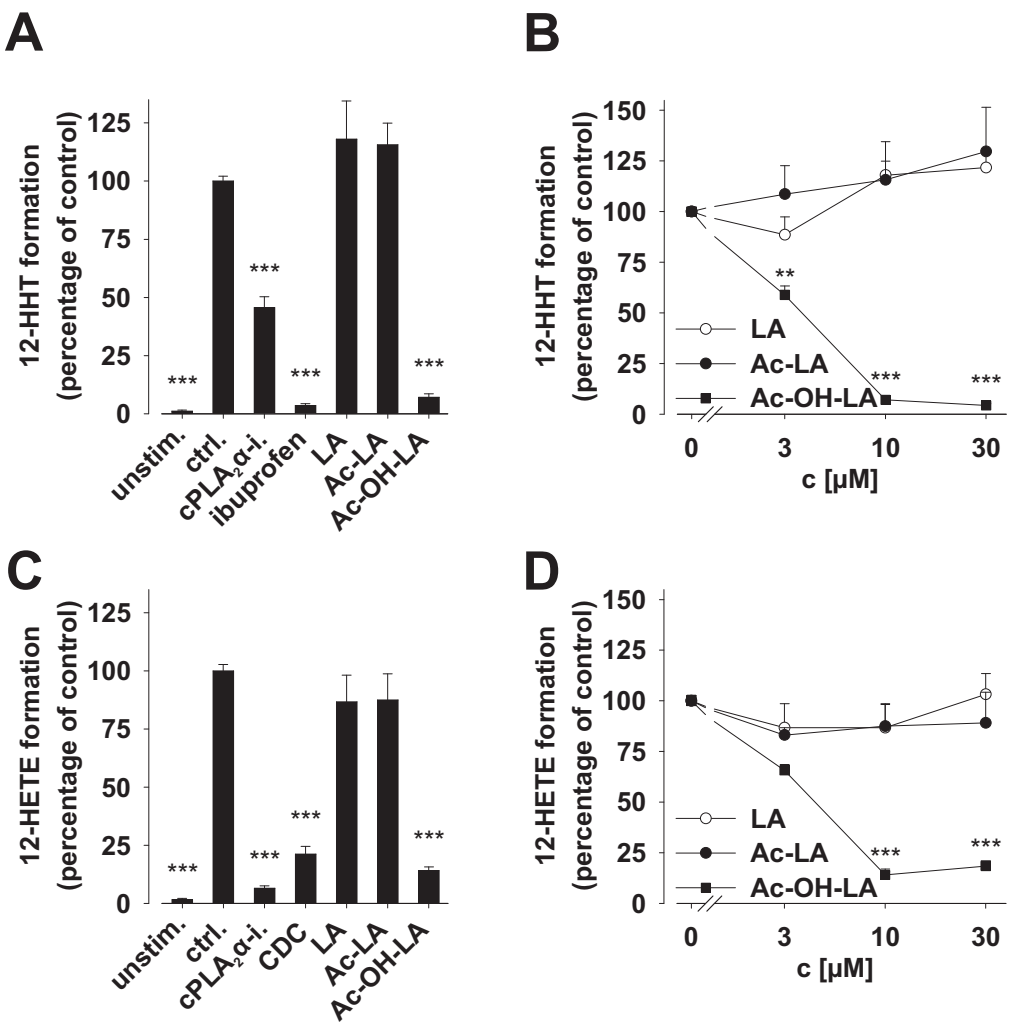


Figure 13: Effects of LAs on A23187-induced 12-HHT and 12-HETE formation in human platelets. Platelets (10^8 /ml in PGC buffer) were pre-incubated with vehicle (ctrl., DMSO), 15 µM cPLA$_2$α inhibitor (cPLA$_2$α-i.), 30 µM ibuprofen, 10 µM CDC , LA, Ac-LA and Ac-OH-LA (10 µM in panels **A** and **C** and at the indicated concentrations in panels **B** and **D**) for 15 min at 37 °C. The cells were stimulated with A23187 1 µM for 5 min at 37 °C. The reaction was terminated and 12-HHT (**A** and **B**) and 12-HETE formation (**C** and **D**) were determined. Data are given as mean + S.E. of the percentage of the vehicle control with 91.0 ± 13.8 ng 12-HHT and 271 ± 34.1 ng 12-HETE per 10^8 cells, n = 3 – 4, ** $p < 0.01$, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey-Kramer *post hoc* test.

Also in PMNL, A23187 led to extensive production of 5-LO products (5(S)-HETE, 5(S),12(S)-diHETE, LTB$_4$ and its all-trans isomers) (Figure **14 A** and **B**), 12-HETE (Figure **14 C** and **D**) and 15-HETE (data not shown). The production of these metabolites was strongly dependent on cPLA$_2$α as it was totally blocked by the control inhibitor cPLA$_2$α-i. Ac-OH-LA completely prevented the metabolite production at a concentration of 10 µM as well. Of interest, LA partly inhibited 5-LO product formation (79.9% inhibition, Figure **14 A** and **B**) without affecting the production of 12- or 15-HETE. 12- and 15-HETE formation were rather stimulated by the LAs lacking the 28-OH group (and the 5-LO control inhibitor BWA4C), which can be attributed to increased AA supply, resulting from inhibitory effects on other AA-metabolizing enzymes.

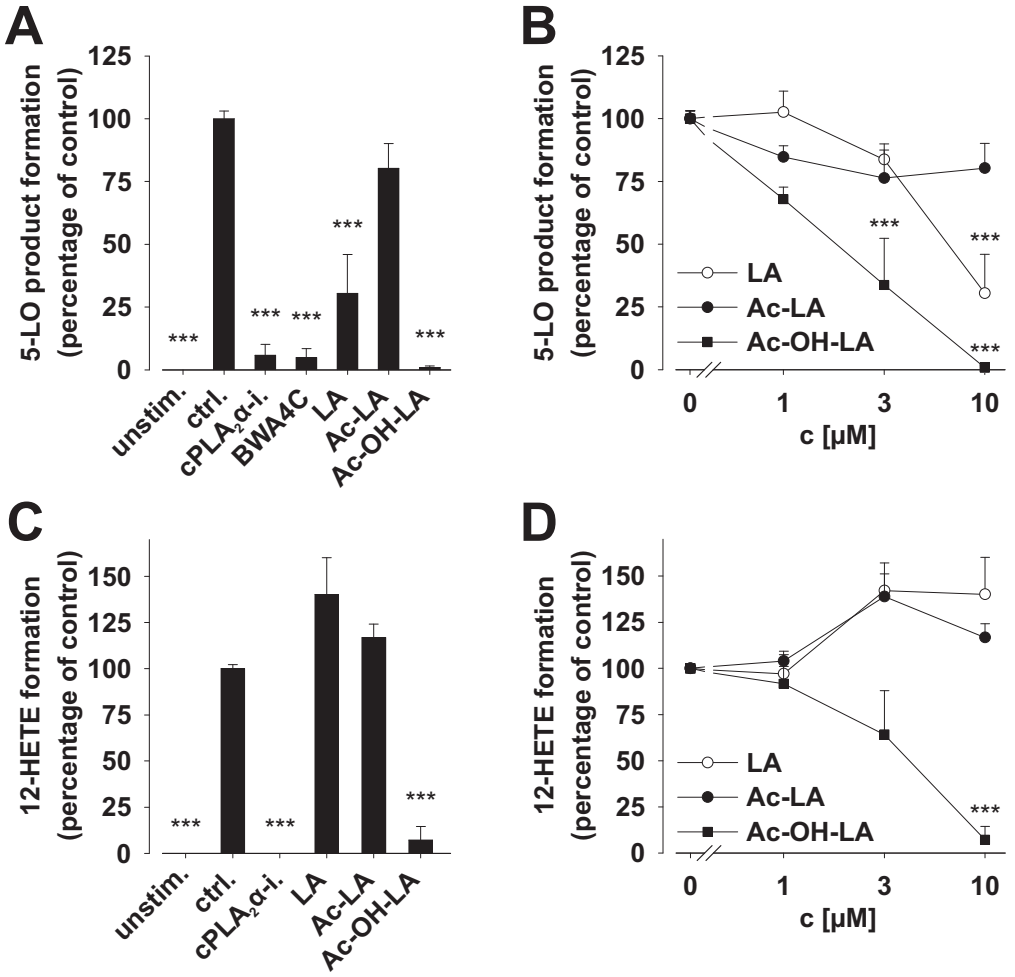


Figure 14: Effects of LAs on A23187-induced 5-LO product and 12-HETE formation in PMNL. PMNL (5×10^6/ml in PGC buffer) were pre-incubated with vehicle (ctrl., DMSO), 5 µM cPLA$_2$α inhibitor (cPLA$_2$α-i.), 0.3 µM BWA4C, LA, Ac-LA and Ac-OH-LA (10 µM each in panels **A** and **C** and at the indicated concentrations in panels **B** and **D**) for 15 min at 37 °C. The cells were stimulated with 1 µM A23187 for 5 min at 37 °C. The reaction was terminated and 5-LO product formation (5(S)-HETE, 5(S),12(S)-diHETE, LTB$_4$ and its all-trans isomers, **A** and **B**) and 12-HETE formation (**C** and **D**) were determined. Data are given as mean + S.E. of the percentage of the vehicle control with 177 ± 18.4 ng 5-LO products and 30.9 ± 9.75 ng 12-HETE per 5×10^6 cells, n = 3, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey HSD *post hoc* test.

Similarly, A23187 provoked metabolite production in monocytes solely dependent on cPLA$_2$α. Like the cPLA$_2$α control inhibitor, Ac-OH-LA (10 µM) completely inhibited the production of 5-LO metabolites (5(S)-HETE, 5(S),12(S)-diHETE, LTB$_4$ and its all-trans isomers, Figure **15 A** and **B**) as well as 12-HETE (Figure **15 C** and **D**), 12-HHT (Figure **15 E** and **F**) and 15-HETE (data not shown). LAs lacking the 28-OH group did not affect the metabolite production except for the 5-LO products. Especially LA inhibited 5-LO product formation, but was still less effective than Ac-OH-LA.

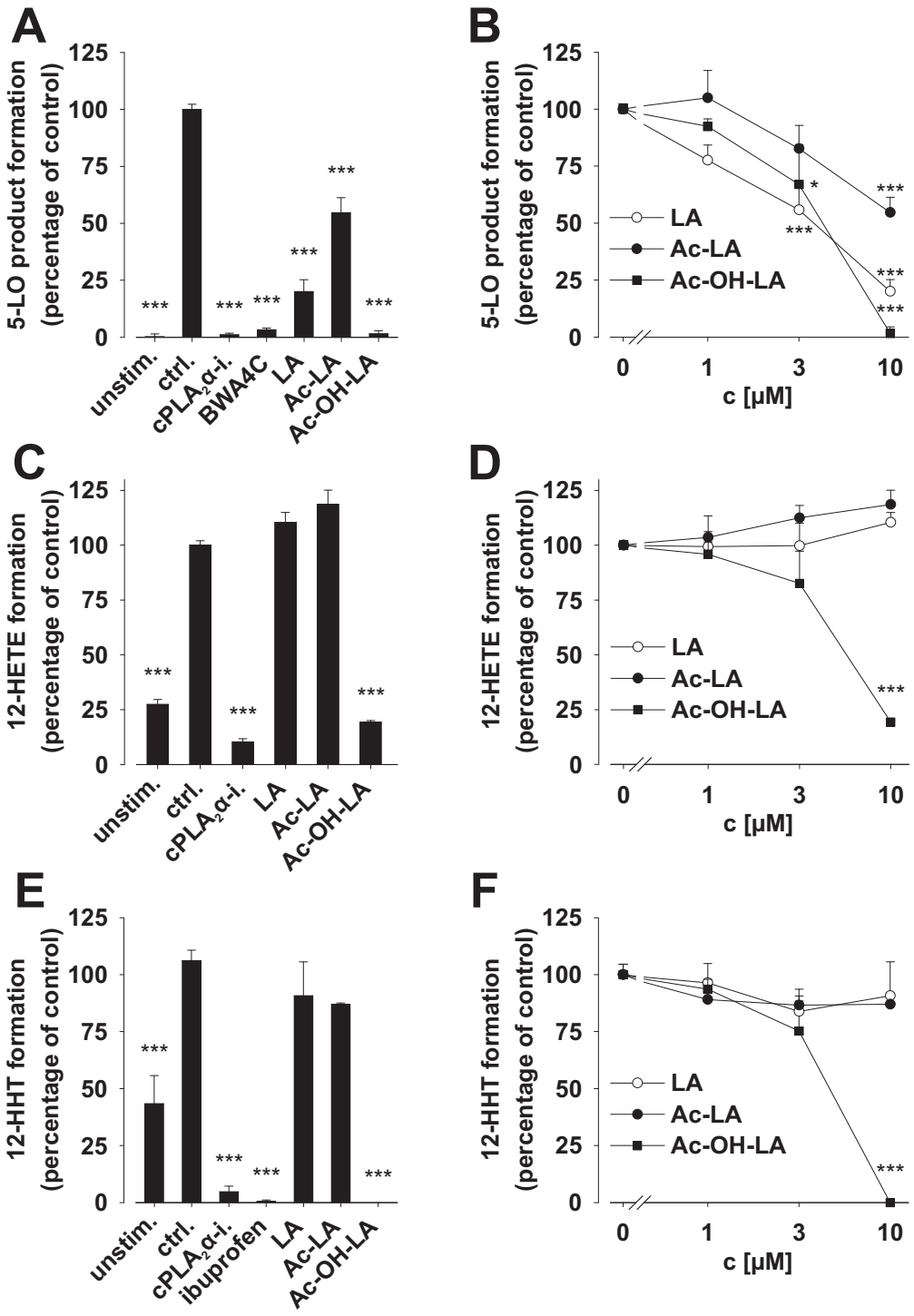


Figure 15: Effects of LAs on A23187-induced 5-LO product, 12-HETE and 12-HHT formation in human monocytes. Monocytes (2×10^6/ml in PGC buffer) were pre-incubated with vehicle (ctrl., DMSO), 5 µM $cPLA_2\alpha$ inhibitor ($cPLA_2\alpha$-i.), 0.3 µM BWA4C, 30 µM ibuprofen, LA, Ac-LA and Ac-OH-LA (10 µM each in panels **A**, **C** and **E** and at the indicated concentrations in panels **B**, **D** and **F**) for 15 min at 37 °C. The cells were stimulated with 1 µM A23187 for 5 min at 37 °C. The reaction was terminated and 5-LO product formation (5(S)-HETE, 5(S),12(S)-diHETE, LTB_4 and its all-trans isomers, **A** and **B**), 12-HETE (**C** and **D**) and 12-HHT (**E** and **F**) formation were determined. Data are given as mean + S.E. of the percentage of the vehicle control with 82.9 ± 16.1 ng 5-LO products, 189 ± 23.4 ng 12-HETE and 31.5 ± 3.66 ng 12-HHT per 2×10^6 cells, n = 3, * $p < 0.05$, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey HSD *post hoc* test.

4.1.4 Effects of lupeolic acids on arachidonic acid metabolite formation in human blood cells after stimulation with exogenous arachidonic acid

To trace the effects of Ac-OH-LA back to the inhibition of AA supply, platelets, PMNL and monocytes were pre-treated with LAs and stimulated with exogenous AA (alone or combined with A23187). The supply of exogenous AA is supposed to circumvent the necessity of endogenous AA release for the generation of 5-, 12-, and 15-LO products as well as COX metabolites.

In platelets, stimulation with AA led to a strong loss of the inhibitory effect of Ac-OH-LA. Thus, 12-HHT production was only moderately affected after pre-treatment with Ac-OH-LA (at 30 µM, 52.2% inhibition after AA stimulation vs. 95.6% after stimulation with A23187, Figure **16 A** and **B**). The 12-HETE production was nearly unaffected after pre-treatment with Ac-OH-LA (Figure **16 C** and **D**). A similar pattern was observed for the cPLA$_2$α control inhibitor that led to about 50% inhibition of 12-HHT formation, but rather stimulated 12-HETE formation.

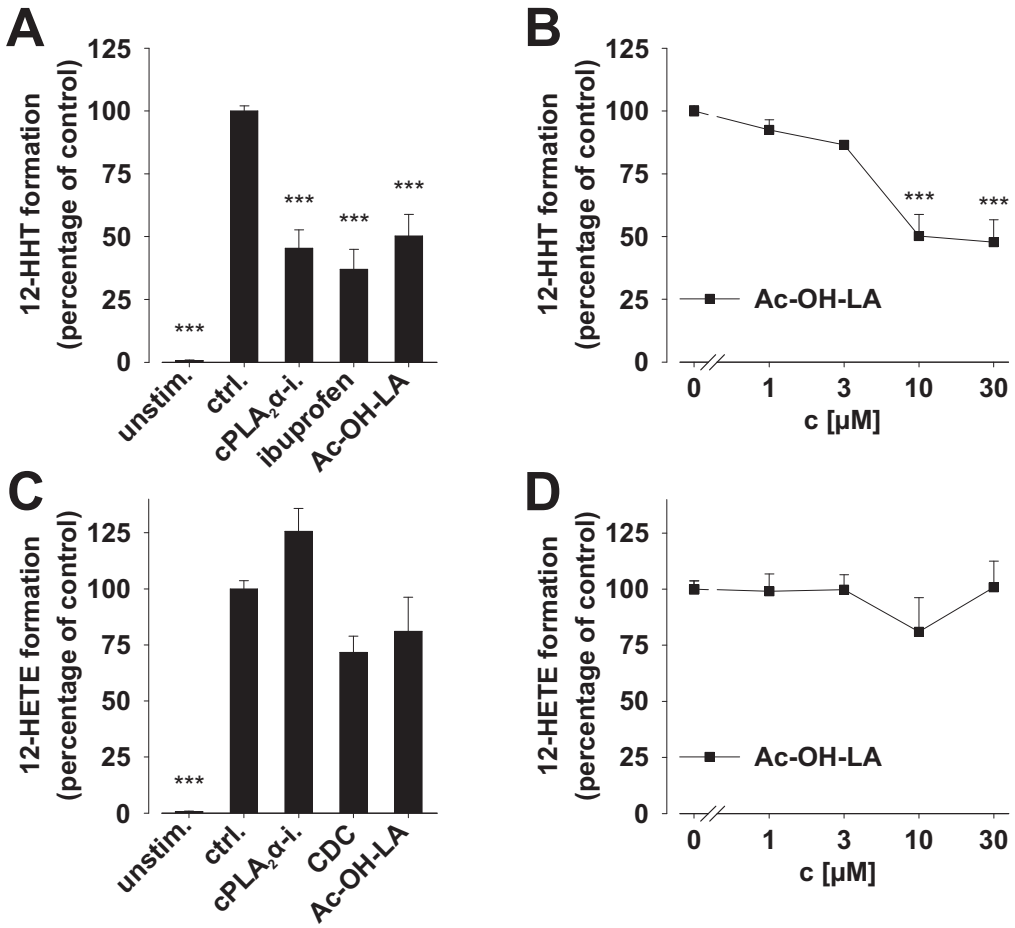


Figure 16: Effects of Ac-OH-LA on AA-induced metabolite formation in human platelets. Platelets (10^8/ml in PGC buffer) were pre-incubated with vehicle (ctrl., DMSO), 15 µM cPLA$_2$α inhibitor (cPLA$_2$α-i.), 30 µM ibuprofen or Ac-OH-LA (10 µM in panels **A** and **C** and at the indicated concentrations in panels **B** and **D**) for 15 min at 37 °C. The cells were stimulated with 5 µM AA for 5 min at 37 °C. The reaction was terminated and 12-HHT (**A** and **B**) and 12-HETE formation (**C** and **D**) were determined. Data are given as mean + S.E. of the percentage of the vehicle control with 170 ± 18.4 ng 12-HHT and 1,052 ± 70.7 ng 12-HETE per 10^8 cells, n = 3 – 6, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey-Kramer *post hoc* test.

In PMNL, supplementation of exogenous AA only partially reconstituted 5-LO product formation after pre-treatment with LA and Ac-OH-LA (Figure **17 A** and **B**). Of interest, also the cPLA$_2$α inhibitor suppressed 5-LO product formation in this approach. In contrast, 12-HETE (Figure **17 C** and **D**) and 15-HETE formation (data not shown) was fully restored in the presence of exogenous AA.

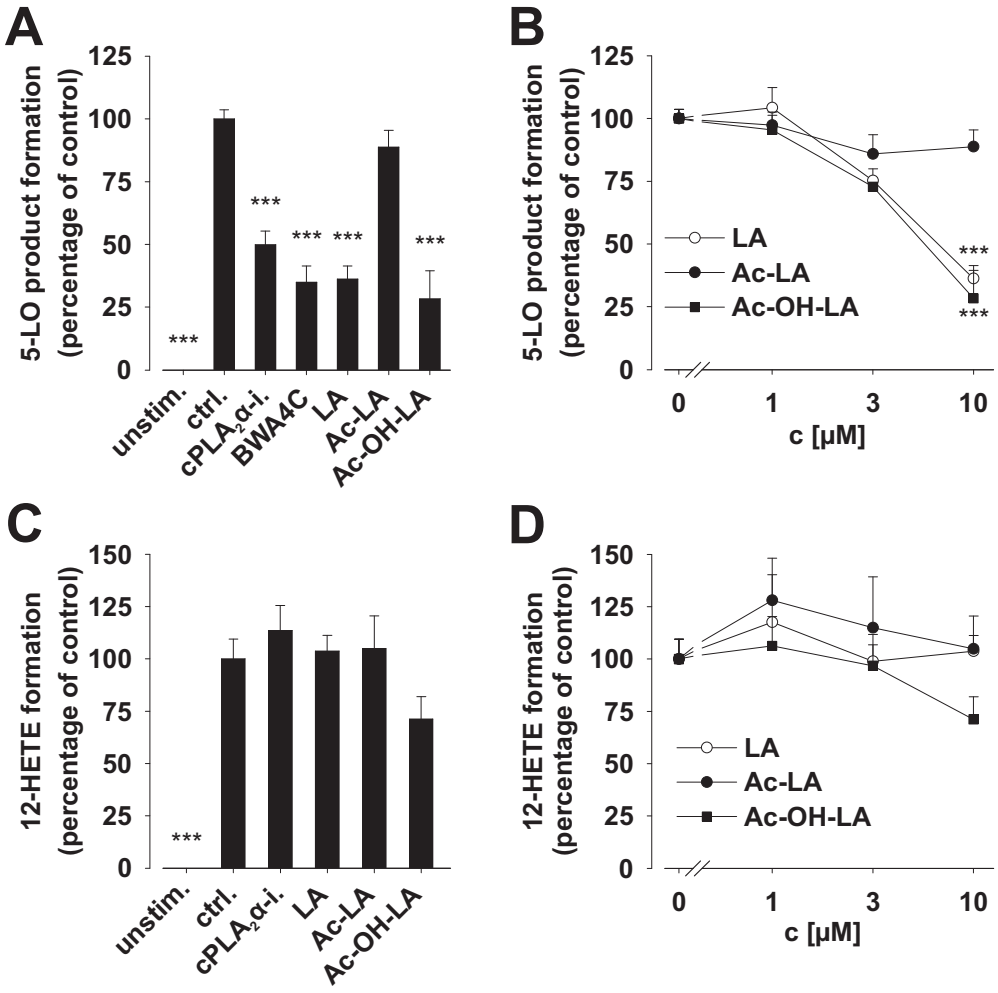


Figure 17: Effects of LAs on AA- plus A23187-induced 5-LO product and 12-HETE formation in human PMNL. PMNL (5×10^6 /ml in PGC buffer) were pre-incubated with vehicle (ctrl., DMSO), 5 µM cPLA$_2$α inhibitor (cPLA$_2$α-i.), 0.3 µM BWA4C, LA, Ac-LA or Ac-OH-LA (10 µM each in panels **A** and **C** and at the indicated concentrations in panels **B** and **D**) for 15 min at 37 °C. The cells were stimulated with 20 µM AA plus 2.5 µM A23187 for 10 min at 37 °C. The reaction was terminated and 5-LO product formation (5(S)-HETE, 5(S),12(S)-diHETE, LTB$_4$ and its all-trans isomers, **A** and **B**) and 12-HETE formation (**C** and **D**) were determined. Data are given as mean + S.E. of the percentage of the vehicle control with 988 ± 163 ng 5-LO products and 129 ± 28.9 ng 12-HETE per 5×10^6 cells, n = 3 – 4, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey-Kramer *post hoc* test.

The same pattern was observed for monocytes. Here, LA and Ac-OH-LA partly retained their inhibitory potential on 5-LO product formation after addition of exogenous AA as well (Figure **18 A** and **B**). In addition, Ac-OH-LA impaired 12-HHT formation at higher concentrations (Figure **18 E** and **F**). On the other hand, 12-HETE (Figure **18 C** and **D**) and 15-HETE (data not shown) formation in Ac-OH-LA-treated monocytes was totally restored after supplementation of AA.

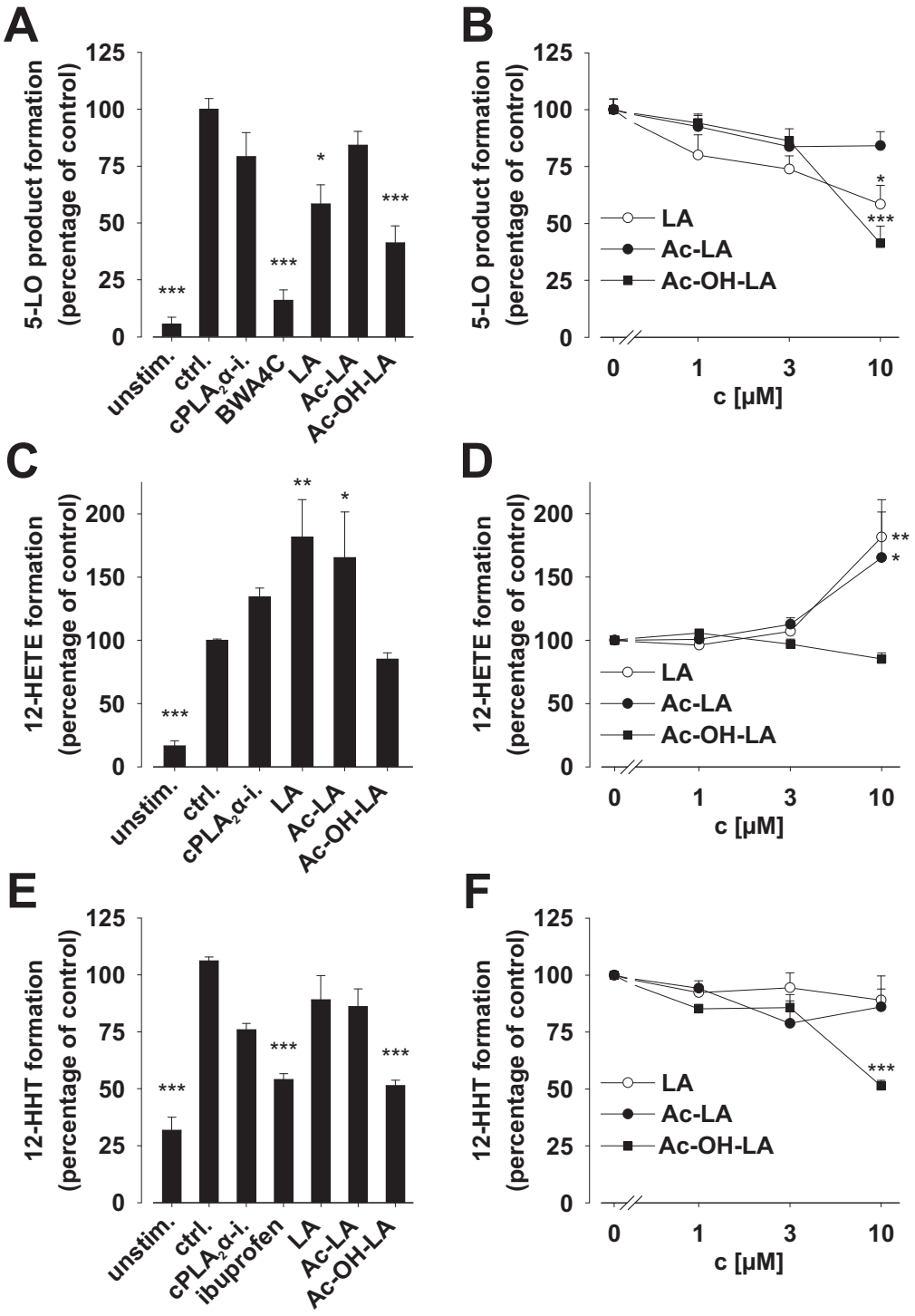


Figure 18: Effects of LAs on AA- plus A23187-induced 5-LO product, 12-HETE and 12-HHT formation in human monocytes. Monocytes (2×10^6 /ml in PGC buffer) were pre-incubated with vehicle (ctrl., DMSO), 5 µM cPLA$_2$α inhibitor (cPLA$_2$α-i.), 0.3 µM BWA4C, 30 µM ibuprofen, LA, Ac-LA or Ac-OH-LA (10 µM each in panels **A**, **C** and **E** and at the indicated concentrations in panels **B**, **D** and **F**) for 15 min at 37 °C. The cells were stimulated with 20 µM AA plus 1 µM A23187 for 5 min at 37 °C. The reaction was terminated and 5-LO product formation (5(S)-HETE, 5(S),12(S)-diHETE, LTB$_4$ and its all-trans isomers, **A** and **B**), 12-HETE (**C** and **D**) and 12-HHT (**E** and **F**) formation were determined. Data are given as mean + S.E. of the percentage of the vehicle control with 44.0 ± 4.79 ng 5-LO products, 183 ± 40.8 ng 12-HETE and 69.3 ± 5.30 ng 12-HHT per 2×10^6 cells, n = 3, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey HSD *post hoc* test.

In conclusion, the inhibitory potential of Ac-OH-LA on AA release by cPLA$_2$α is obviously influencing the production of AA metabolites in human blood cells. Nevertheless, additional effects (e.g. inhibition of 5-LO or COX enzymes) contribute to the overall inhibition of metabolite production.

4.1.5 Collagen-induced, arachidonic acid-dependent platelet aggregation is influenced by 3-*O*-acetyl-28-hydroxy-lupeolic acid

Human platelets are commonly stimulated by collagen after injury of blood vessels. Collagen binds to its receptor glycoprotein VI on the platelet's membrane, and leads to activation of cPLA$_2$α via phosphorylation cascades. The liberated AA is subsequently converted to PGH$_2$ by COX-1 and then to TXA$_2$ by thromboxane synthase [577]. TXA$_2$ binds to its G protein-coupled receptor TPα on platelet membranes which leads to activation of phospholipase C (PLCβ) [578]. Thereby it activates protein kinase C (PKC) and induces Ca^{2+} release that finally leads to platelet aggregation [577-578]. Another way to activate platelet aggregation is the direct stimulation with TXA$_2$ or its stable analogue U46619, which does not include cPLA$_2$α activation [577]. To investigate functional effects of LAs on cPLA$_2$α activity in a cellular system, platelets were pre-incubated with LAs (15 min, 37 °C) and stimulated with the stimuli U46619 or collagen. After stimulation with U46619, the control inhibitors indomethacin and cPLA$_2$α-i. were ineffective as expected (Figure **19 A**), highlighting the independence of U46619-induced platelet aggregation from the COX-1-cPLA$_2$α axis. On the other hand, collagen-induced platelet aggregation was totally prevented by the control inhibitor indomethacin that inhibits TXA$_2$ formation through inhibition of COX-1, (Figure **19 B**). cPLA$_2$α-i. reduced collagen-induced platelet aggregation to a large extent (84% reduction compared to the vehicle control) as well.

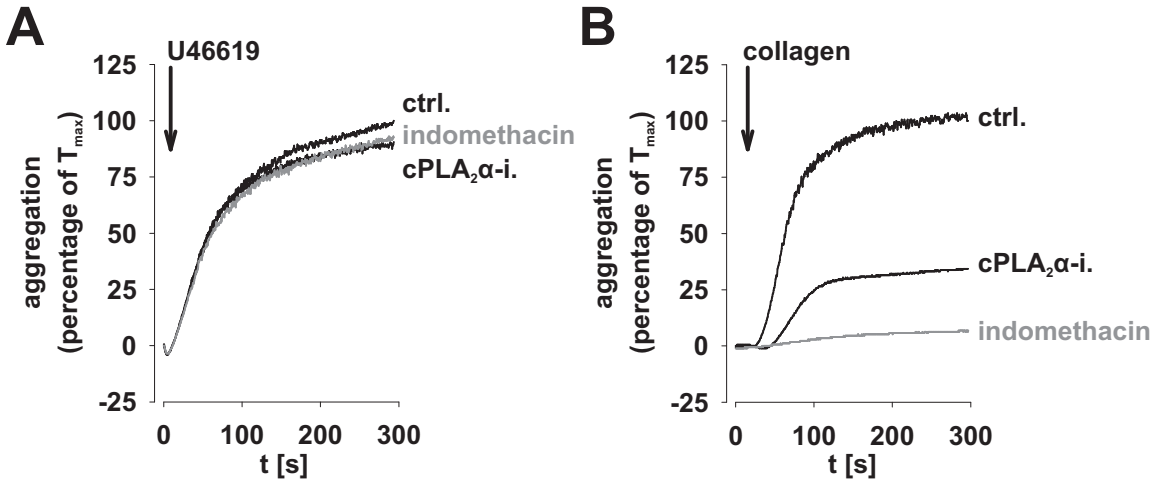


Figure 19: Effects of cPLA$_2$α-i. and indomethacin on U46619- and collagen-induced platelet aggregation. Platelets (2×10^8 /ml in tyrode's buffer) were pre-incubated with vehicle (ctrl., DMSO), 5 µM cPLA$_2$α-i. or 20 µM indomethacin for 15 min at 37 °C, then supplemented with $CaCl_2$ (1 mM) and stimulated with 1 µM U46619 (**A**) or 0.6 µg/ml collagen (**B**). Light transmission was recorded over 5 min. Data are shown as percentage of the maximum aggregation after vehicle pre-incubation and stimulation with the appropriate stimulus. Results are representative for at least three experiments.

Pre-treatment of platelets with Ac-OH-LA hardly affected U46619-induced platelet aggregation up to 10 µM (Figure **20 A**) but led to considerable inhibition of collagen-induced aggregation at this concentration (Figure **20 B**). Collagen-induced platelet aggregation was almost totally blocked by 30 µM Ac-OH-LA, but also U46619-induced aggregation was partly affected at this concentration, pointing to an additional non-cPLA$_2$α-dependent effect.

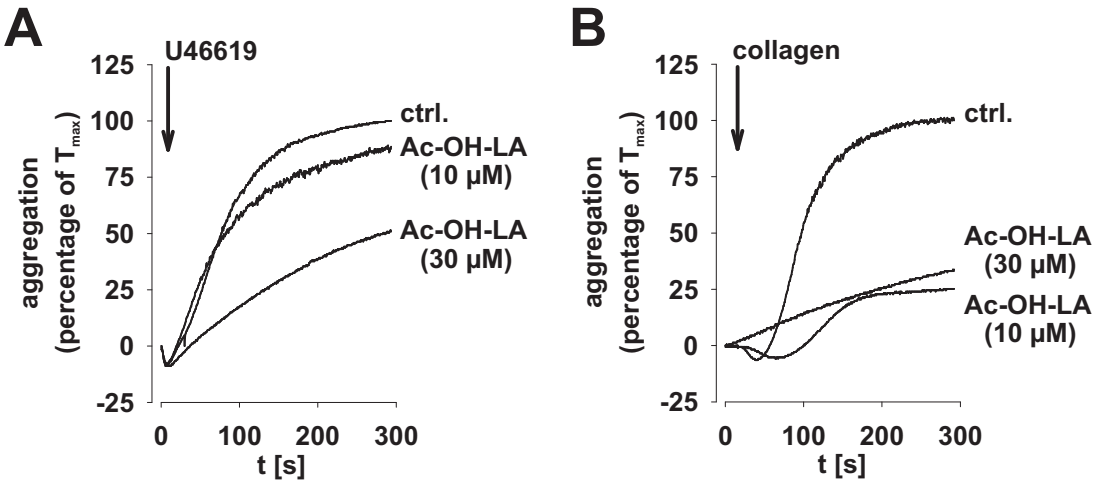


Figure 20: Effects of Ac-OH-LA on U46619- and collagen-induced platelet aggregation. Platelets (2×10^8 /ml in tyrode's buffer) were pre-incubated with vehicle (ctrl., DMSO) or Ac-OH-LA (10 µM and 30 µM) for 15 min at 37 °C, then supplemented with $CaCl_2$ (1 mM) and stimulated with 1 µM U46619 (**A**) or 0.6 µg/ml collagen (**B**). Light transmission was recorded over 5 min. Data are shown as percentage of the maximum aggregation after vehicle pre-incubation and stimulation with the appropriate stimulus. Results are representative for at least three experiments.

The two LAs that are not hydroxylated in the C-28 position showed an unexpected behaviour in this test system. In fact, LA and Ac-LA at 10 and 30 µM totally blocked platelet aggregation irrespective of the stimulus (collagen (Figure **21 B** and **D**) or U46619 (Figure **21 A** and **C**)).

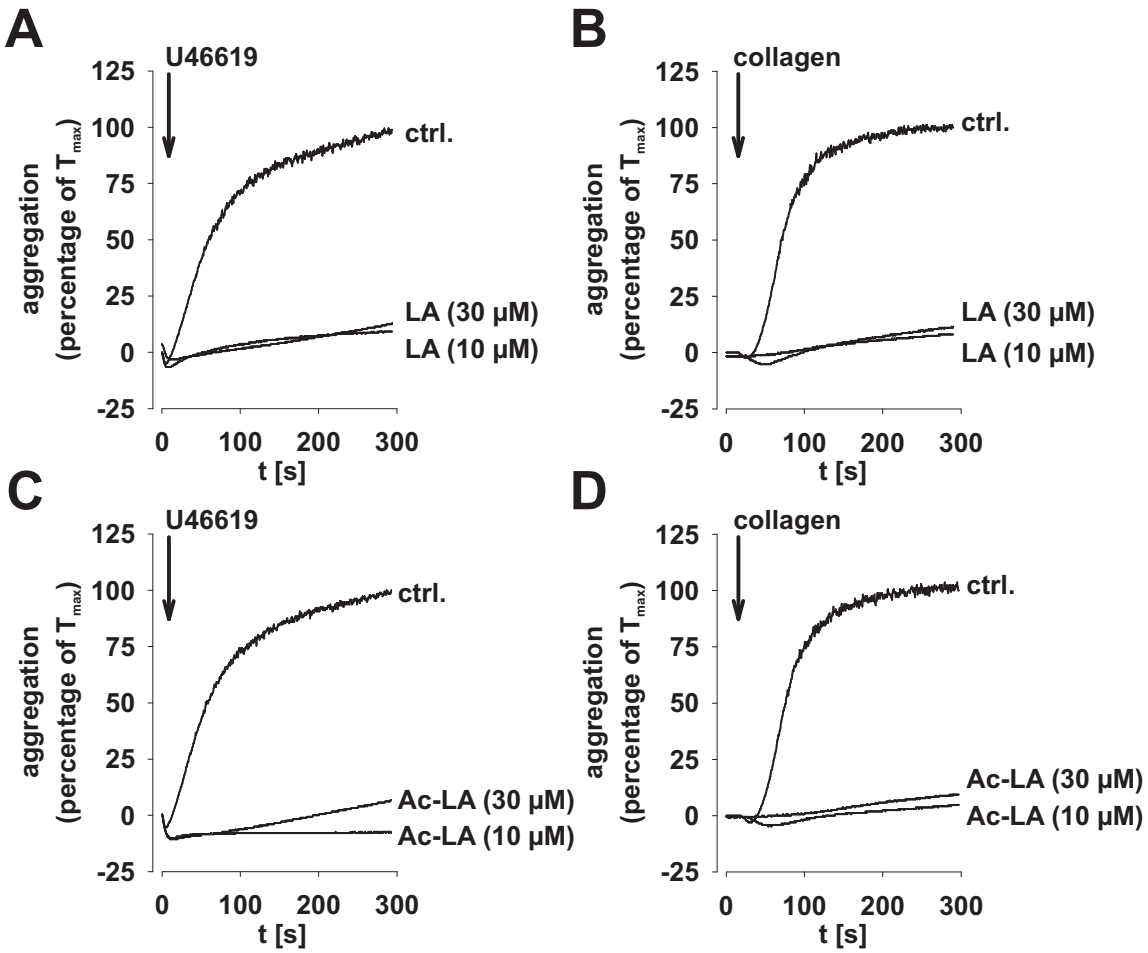


Figure 21: Effects of LAs lacking the 28-OH group on U46619- and collagen-induced platelet aggregation. Platelets (2×10^8 /ml in tyrode's buffer) were pre-incubated with vehicle (ctrl., DMSO), LA (10 µM and 30 µM, panels **A** and **B**) or Ac-LA (10 µM and 30 µM, panels **C** and **D**) for 15 min at 37 °C, then supplemented with $CaCl_2$ (1 mM) and stimulated with 1 µM U46619 (**A** and **C**) or 0.6 µg/ml collagen (**B** and **D**). Light transmission was recorded over 5 min. Data are shown as percentage of the maximum aggregation after vehicle pre-incubation and stimulation with the appropriate stimulus. Results are representative for at least three experiments.

Concentration-response analyses revealed an IC_{50} value of about 8 µM for Ac-OH-LA in collagen-induced platelet aggregation (Figure **22 B**). At this concentration, Ac-OH-LA did not yet affect U46619-induced aggregation (Figure **22 A**). In contrast, LA showed an IC_{50} value of about 3 µM after stimulation with either collagen or U46619. For Ac-LA the IC_{50} value after stimulation with U46619 was about 3 µM as well, whereas the IC_{50} value after stimulation with collagen shifted to about 1 µM. This suggests two different effects of LAs on platelet aggregation, one taking action upstream and the other one downstream of TXA_2 synthesis.

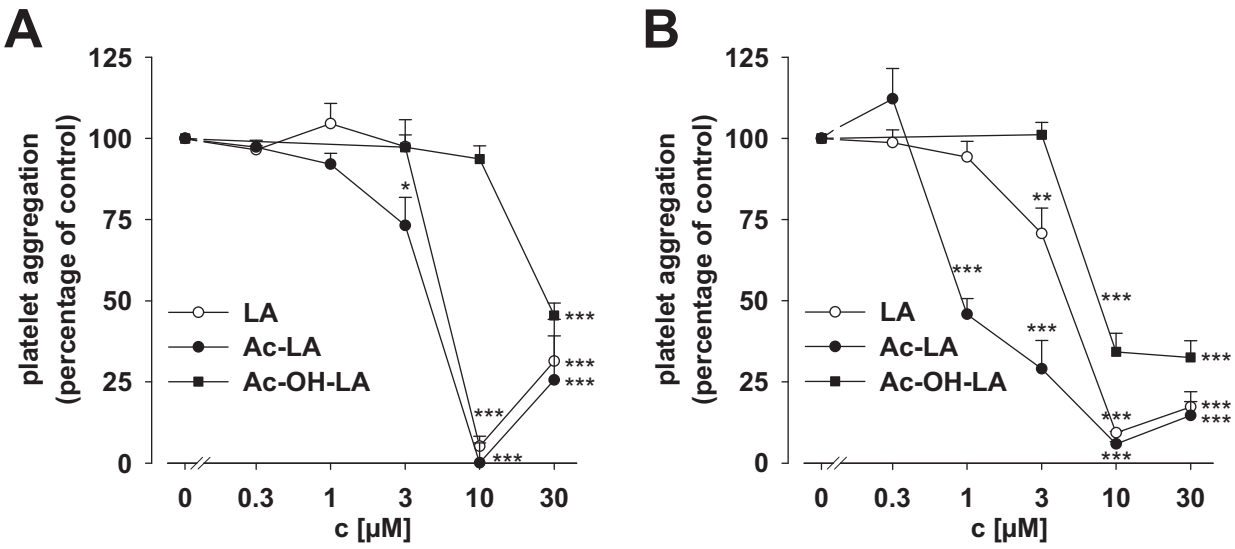


Figure 22: Effects of LAs on U46619- and collagen-induced platelet aggregation. Platelets (2×10^8 /ml in tyrode's buffer) were pre-incubated with vehicle (ctrl., DMSO) or LAs at the respective concentrations for 15 min at 37 °C, then supplemented with $CaCl_2$ (1 mM) and stimulated with 1 µM U46619 (**A**) or 0.6 µg/ml collagen (**B**). Data are given as mean + S.E. as percentage related to the vehicle-treated controls after stimulation with the appropriate stimulus for 5 min. n = 3 – 5, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey-Kramer *post hoc* test.

4.1.6 Effects of lupeolic acids on arachidonic acid metabolite production in A23187-stimulated human whole blood

In order to analyze the action of LAs on cPLA$_2$α in a more physiological test system, human whole blood was pre-treated with LA or Ac-OH-LA and stimulated with A23187 (30 µM) to induce Ca^{2+}-triggered mobilization of AA through cPLA$_2$α. The formation of 5-LO products (Figure **23 A**), the 12-LO product 12-HETE (Figure **23 B**) and the COX product 12-HHT (Figure **23 C**) were analyzed. As expected, the control inhibitors BWA4C, CDC and ibuprofen clearly repressed the production of the respective AA metabolites. Surprisingly, Ac-OH-LA as well as LA did not impair the formation of any investigated metabolite at concentrations of 10 or 30 µM.

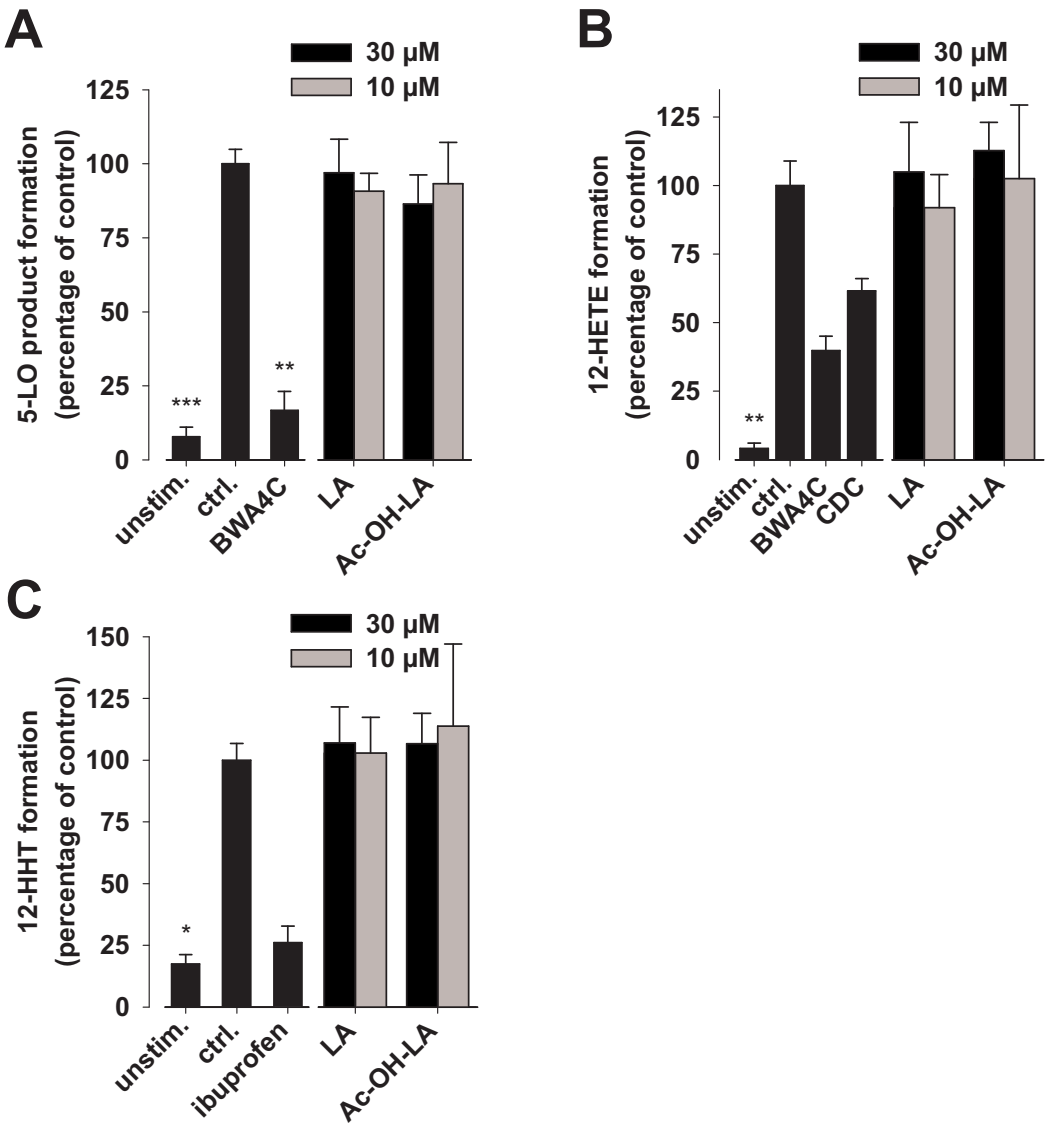


Figure 23: Effects of LAs on arachidonic acid metabolite production in human whole blood. Heparinized human whole blood was pre-incubated with vehicle (ctrl., DMSO), 3 µM BWA4C, 30 µM CDC, 100 µM ibuprofen, LA or Ac-OH-LA (10 and 30 µM) for 10 min at 37 °C. Samples were stimulated with A23187 (30 µM) for 10 min at 37 °C, the incubation was stopped on ice and the amount of 5-LO products 5(S)-HETE, 5(S),12(S)-diHETE and LTB_4 and its all-trans isomers (**A**) as well as the 12-LO product 12-HETE (**B**) and the COX product 12-HHT (**C**) were determined. Data are given as mean + S.E. of the percentage of the vehicle control with 166 ± 50.0 ng 5-LO products, 485 ± 177 ng 12-HETE and 99.0 ± 26.3 ng 12-HHT per ml blood, n = 3, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey HSD *post hoc* test.

4.1.7 Effects of 3-*O*-acetyl-28-hydroxy-lupeolic acid on cPLA$_2$α activity are impaired by supplementation of BSA

The failure of test compounds in whole blood assays, despite the potent inhibition of certain pharmacological targets in non-cellular test systems or in isolated cells, is frequently associated with an increased tendency of these compounds to undergo protein binding. Accordingly, LAs may interact with albumin leading to strong albumin-binding. The inhibitory potential of Ac-OH-LA on cPLA$_2$α activity in a cell-free model was attenuated after supplementation with

1 mg/ml BSA (Figure **24 A**) compared to samples without BSA (Figure **24 B**). Even though the difference was statistically not significant, after addition of BSA the IC_{50} value was approximately twice as high.

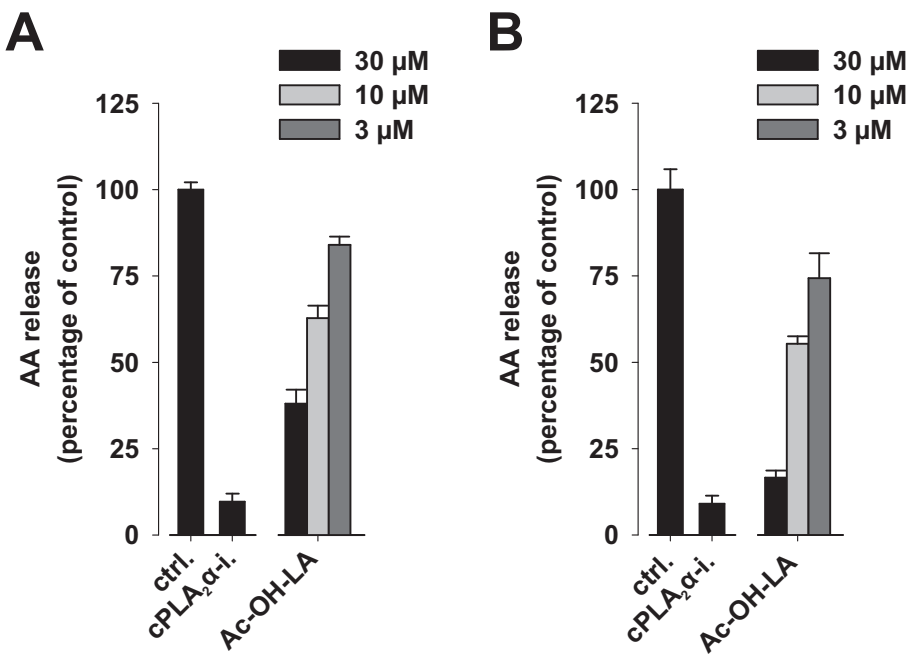


Figure 24: Effects of albumin on the inhibitory potency of Ac-OH-LA in cPLA$_2$α-mediated AA release. PAPC/POG-vesicles (lipid concentration 250 µM in TBS buffer with (1 mg/ml, **A**) or without FAF-BSA (**B**)) were supplemented with $CaCl_2$ (1 mM) and pre-incubated with vehicle (ctrl., DMSO), 5 µM cPLA$_2$α inhibitor (cPLA$_2$α-i.) or Ac-OH-LA at the indicated concentrations for 10 min at RT. The reaction was started by addition of the purified cPLA$_2$α enzyme (2.5 µg/ml) and maintained at 37 °C for 60 min. After derivatization, AA was analyzed by HPLC. Data are given as mean + S.E. of the percentage of the vehicle control, n = 3 – 7.

To summarize, Ac-OH-LA, a lupeolic acid derivative that is hydroxylated in the C-28 position, acts as a potent inhibitor of cPLA$_2$α. This was demonstrated in a cell-free test system as well as in cell-based assays using different cell types. The inhibition of cPLA$_2$α leads to diminished production of AA metabolites in these cells, and thereby results in functional cellular effects. However, the effect of Ac-OH-LA on cPLA$_2$α cannot be observed in human whole blood, which might be attributed to the interaction with plasma proteins.

4.2 Modulation of arachidonic acid mobilization in human platelets by tirucallic acids

TAs were already reported to entail activating properties on 5-LO product formation in neutrophils [538] and these effects were proposed to be responsible for pro-inflammatory effects of frankincense formulations [508]. However, also BAs were found to elicit stimulating properties in neutrophils and platelets [539, 579]. This chapter describes the TA-induced effects on AA release in platelets.

4.2.1 Effects of tirucallic acids on arachidonic acid release in platelets

In resting human platelets, resuspended in Ca^{2+}-containing buffer (1 mM), treatment with TAs (10 µM, 5 min) led to a considerable release of AA, with 3α-OH-TA increasing AA release about threefold vs. vehicle control. 3β-OH-TA and 3-oxo-TA led to similar effects on AA release (Figure **25 A**). Ac-TA was less potent and amplified AA release only about twofold. As expected, calcium ionophore A23187 (1 µM) massively induced AA release in the presence of Ca^{2+}, whereas this stimulation was largely blocked after deprivation of extra- and intracellular Ca^{2+} with EDTA and BAPTA-AM (Figure **25 B**). In contrast, chelation of Ca^{2+} by EDTA and BAPTA-AM did not impair the ability of the TAs (10 µM, 15 min) to induce AA release. Thus, 3α-OH-TA and 3-oxo-TA increased AA liberation about fivefold, 3β-OH-TA and Ac-TA induced AA release about three- and sixfold, respectively.

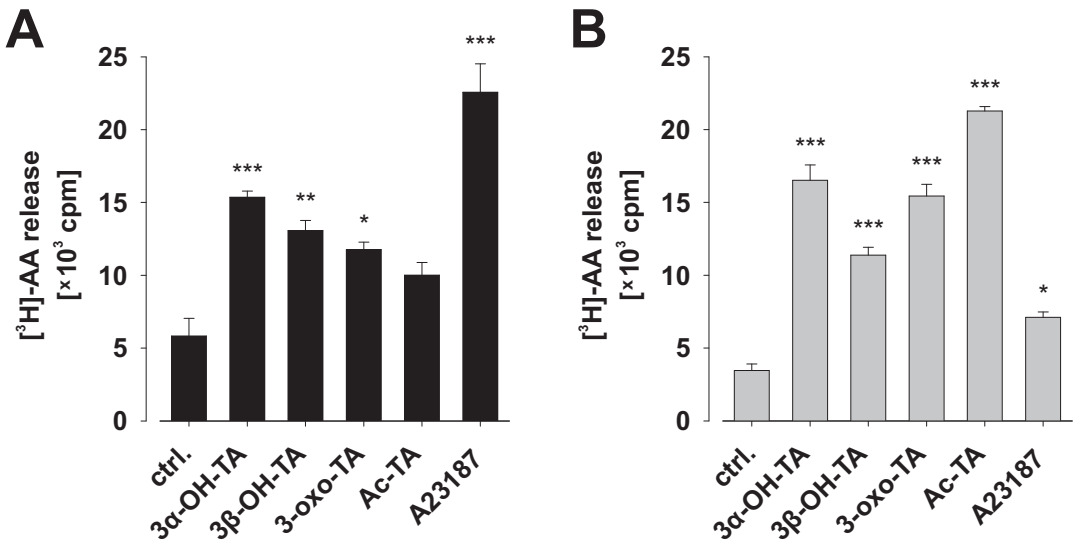


Figure 25: Effect of tirucallic acids on arachidonic acid release in human platelets. [^{3}H]-AA-labelled platelets (10^8 /ml in PG buffer containing 1 mg/ml FAF-BSA) were supplemented with $CaCl_2$ 1 mM (1 min, 37 °C, **A**) or pre-treated with 1 mM EDTA and 30 µM BAPTA-AM for 15 min at RT and were pre-warmed to 37 °C for 1 min (**B**). The samples were stimulated with vehicle (ctrl., DMSO), 3α-OH-TA, 3β-OH-TA, 3-oxo-TA or Ac-TA (10 µM) or A23187 (1 µM) at 37 °C for 5 min (**A**) or 15 min (**B**). The incubation was stopped on ice and cells were spun down. Aliquots of the supernatants were mixed with scintillation cocktail and measured on a scintillation counter to detect released [^{3}H]-AA. Data are given as mean + S.E. in cpm, n = 3, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey HSD *post hoc* test.

4.2.2 Impact of tirucallic acids on arachidonic acid release in PMNL

Treatment of [^{3}H]-AA-labelled human PMNL with TAs (10 µM, 5 min) did not induce AA mobilization. None of the TAs significantly enhanced AA release; though AA mobilization was clearly responsive to stimulation with A23187 (1 µM, Figure **26**).

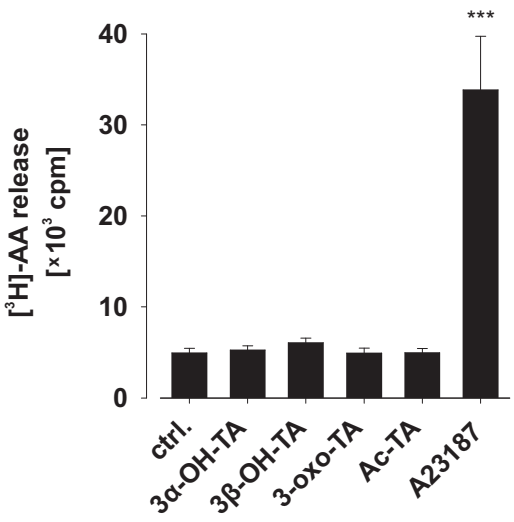


Figure 26: Effect of tirucallic acids on arachidonic acid release in human neutrophils. [^{3}H]-AA-labelled PMNL (2×10^7 /ml in PG buffer) were supplemented with 1 mM $CaCl_2$ (1 min, 37 °C). The samples were stimulated with vehicle (ctrl., DMSO), 3α-OH-TA, 3β-OH-TA, 3-oxo-TA or Ac-TA (10 µM) or A23187 (1 µM) at 37 °C for 5 min. The incubation was stopped on ice and the cells were spun down. Aliquots of the supernatants were mixed with scintillation cocktail and measured on a scintillation counter to detect released [^{3}H]-AA. Data are given as mean + S.E. in cpm, n = 4, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey HSD *post hoc* test.

4.2.3 Arachidonic acid metabolite production in platelets after treatment with tirucallic acids

In human platelets, AA release induced by TAs led to a manifest increase of AA metabolite production. In Ca^{2+}-enriched buffer (1 mM), 12-HHT production was stimulated six- to 20-fold (Figure **27 A** and Table **5**); 12-HETE production was stimulated five- to twelve-fold (Figure **27 C** and Table **5**). After deprivation of Ca^{2+}, the absolute 12-HHT production after TA-treatment declined to about 25 to 50%, but compared to the vehicle control 3α-OH-TA and Ac-TA stimulated 12-HHT production 18- to 40-fold (Figure **27 B** and Table **5**). After 3β-OH-TA and 3-oxo-TA treatment, the relative stimulation of 12-HHT production remained about constant. 12-HETE production was affected most vigorously after Ca^{2+} deprivation: the absolute 12-HETE formation of the vehicle control was largely blocked in the absence of Ca^{2+}, but it remained stable after 3β-OH-TA, 3-oxo-TA, and Ac-TA treatment and was almost doubled after 3α-OH-TA treatment. Compared to the vehicle control, TAs increased 12-HETE formation 45- to 290-fold (Figure **27 D** and Table **5**).

As expected, A23187 (1 µM) led to extensive metabolite production in Ca^{2+}-containing buffer that was about four- to ten-fold more intense than after incubation with TAs. The potency was markedly attenuated after deprivation of Ca^{2+}, but it was not totally blocked.

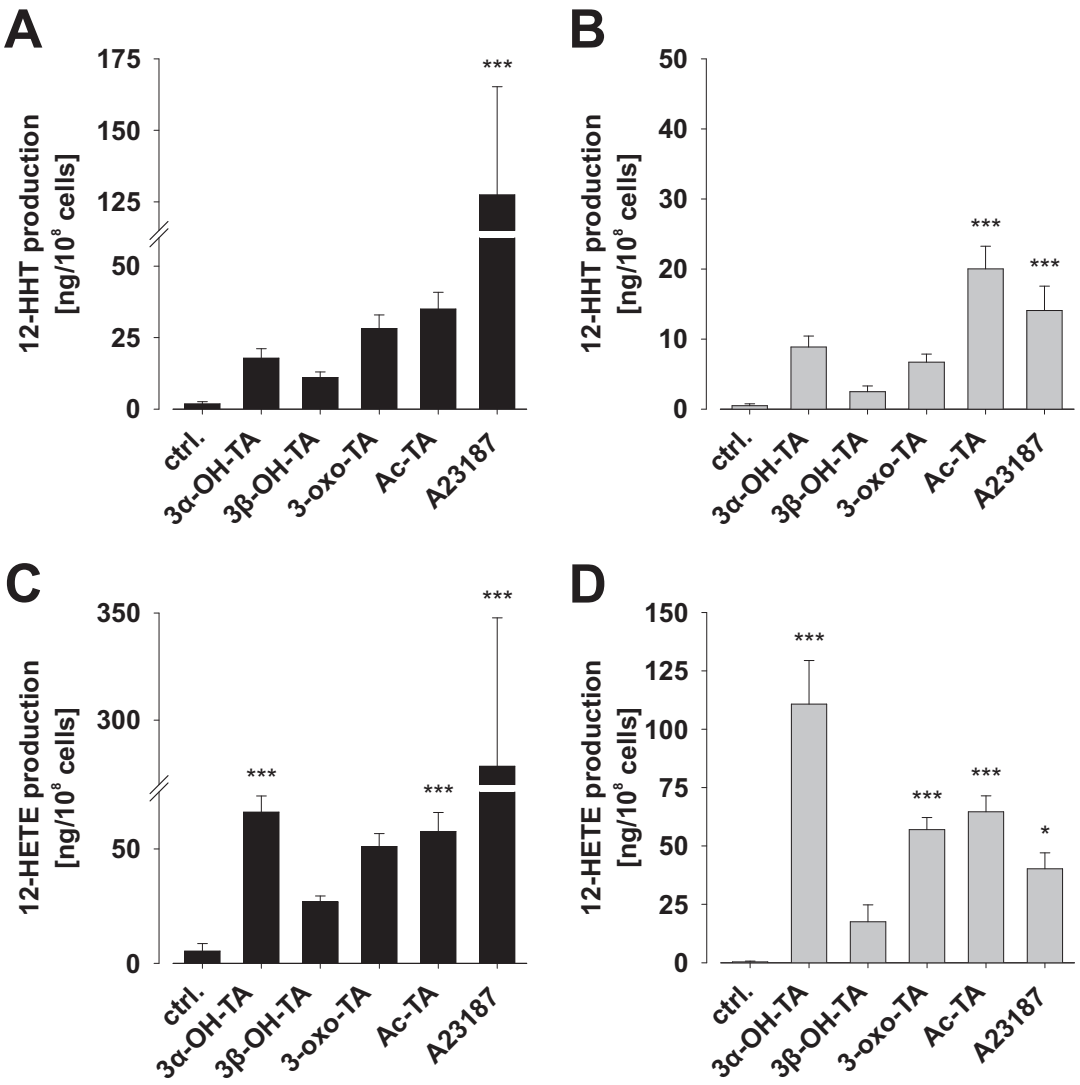


Figure 27: Effect of tirucallic acids on arachidonic acid metabolite production in human platelets. Platelets (10^8 /ml in PG buffer) were supplemented with 1 mM $CaCl_2$ (1 min, 37 °C, panels **A** and **C**) or pre-treated with 1 mM EDTA and 30 µM BAPTA-AM for 15 min at RT and pre-warmed to 37 °C for 1 min (panels **B** and **D**). The samples were incubated with vehicle (ctrl., DMSO), 3α-OH-TA, 3β-OH-TA, 3-oxo-TA or Ac-TA (10 µM) or A23187 (1 µM) at 37 °C for 5 min. The reaction was terminated and 12-HHT (**A** and **B**) and 12-HETE formation (**C** and **D**) were determined. Data are given as mean + S.E. in ng per 10^8 cells. n = 7 – 9, * $p < 0.05$, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey-Kramer *post hoc* test.

Table 5: Absolute and relative production of 12-HHTand 12-HETE after treatment of human platelets with tirucallic acids. Platelets (10^8 /ml in PG buffer) were supplemented with 1 mM $CaCl_2$ (1 min, 37 °C) or pre-treated with 1 mM EDTA and 30 µM BAPTA-AM for 15 min at RT and pre-warmed to 37 °C for 1 min. The samples were incubated with vehicle (ctrl., DMSO), 3α OH-TA, 3β-OH-TA, 3-oxo-TA or Ac-TA (10 µM) or A23187 (1 µM) at 37 °C for 5 min. The reaction was terminated and 12-HHT and 12-HETE formation were determined. Absolute data are given as mean + S.E. in ng per 10^8 cells, relative data are given as proportional value related to the respective vehicle control. n = 7 – 9.

	12-HHT production				12-HETE production			
	Ca^{2+} (1 mM)		EDTA / BAPTA-AM		Ca^{2+} (1 mM)		EDTA / BAPTA-AM	
sample	[ng/10^8 cells]	relative increase vs. ctrl.	[ng/10^8 cells]	relative increase vs. ctrl.	[ng/10^8 cells]	relative increase vs. ctrl.	[ng/10^8 cells]	relative increase vs. ctrl.
ctrl.	1.83 ± 0.74	1	0.48 ± 0.26	1	5.34 ± 3.22	1	0.38 ± 0.29	1
3α-OH-TA	17.9 ± 3.26	9.8	8.85 ± 1.59	18.4	66.1 ± 7.08	12.3	110.8 ± 18.6	289.0
3β-OH-TA	11.0 ± 1.95	6.0	2.48 ± 0.84	5.2	26.9 ± 2.52	5.0	17.6 ± 7.20	45.8
3-oxo-TA	28.2 ± 4.78	15.4	6.71 ± 1.14	14.0	51.0 ± 5.74	9.5	57.0 ± 5.14	148.8
Ac-TA	35.0 ± 5.82	19.2	20.0 ± 3.24	41.7	57.6 ± 8.34	10.8	64.6 ± 6.86	168.7
A23187	127.4 ± 37.8	69.8	14.1 ± 3.49	29.3	278.6 ± 69.1	52.0	40.2 ± 6.89	104.9

Further analysis revealed concentration-dependency of the stimulatory effect of the TAs on 12-HHT and 12-HETE formation (Figure **28 A** and **B**). Significant metabolite production was obtained at 3 µM for Ac-TA. 3α-OH-TA and 3-oxo-TA induced significant AA metabolite production at 10 µM. 3β-OH-TA-induced metabolite production was not significantly elevated by concentrations up to 30 µM but a stimulatory potential was perceptible up from 10 µM.

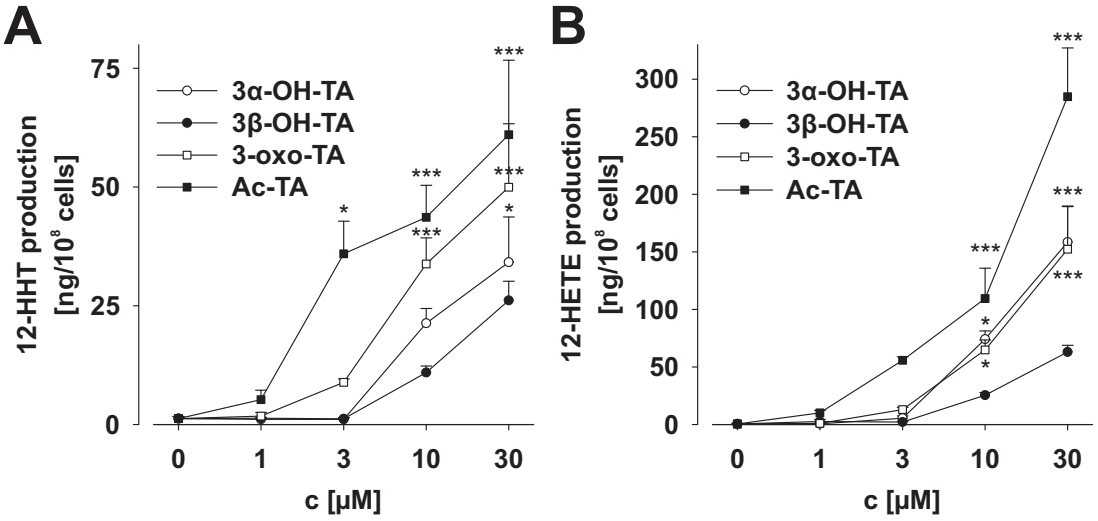


Figure 28: Concentration dependency of arachidonic acid metabolite production in human platelets after treatment with tirucallic acids. Platelets (10^8 /ml in PG buffer) were supplemented with $CaCl_2$ 1 mM (1 min, 37 °C). The samples were incubated with vehicle (DMSO) or 3α-OH-TA, 3β-OH-TA, 3-oxo-TA and Ac-TA at the respective concentrations for 5 min at 37 °C. The reaction was terminated and 12-HHT (**A**) and 12-HETE formation (**B**) were determined. Data are given as mean + S.E. in ng per 10^8 cells, n = 4, * $p < 0.05$, *** $p < 0.001$ vs. vehicle control, one-way ANOVA followed by Tukey-Kramer *post hoc* test.

Incubation of platelets with TAs (10 µM) for time intervals of 30 sec to 60 min revealed a differential pattern of 12-HHT and 12-HETE production. 12-HHT formation was rapidly induced and maximal production was achieved already after 2 min (Figure **29 A**). At later time points, the 12-HHT levels remained constant or even tended to decrease. In contrast, 12-HETE levels continuously increased within 60 min with moderate attenuation of velocity over time (Figure **29 B**). The effects of 3α-OH-TA, 3-oxo-TA and Ac-TA on 12-HHT and 12-HETE formation were almost alike, especially at later time points. In contrast, 3β-OH-TA led to a very modest increase in metabolite production.

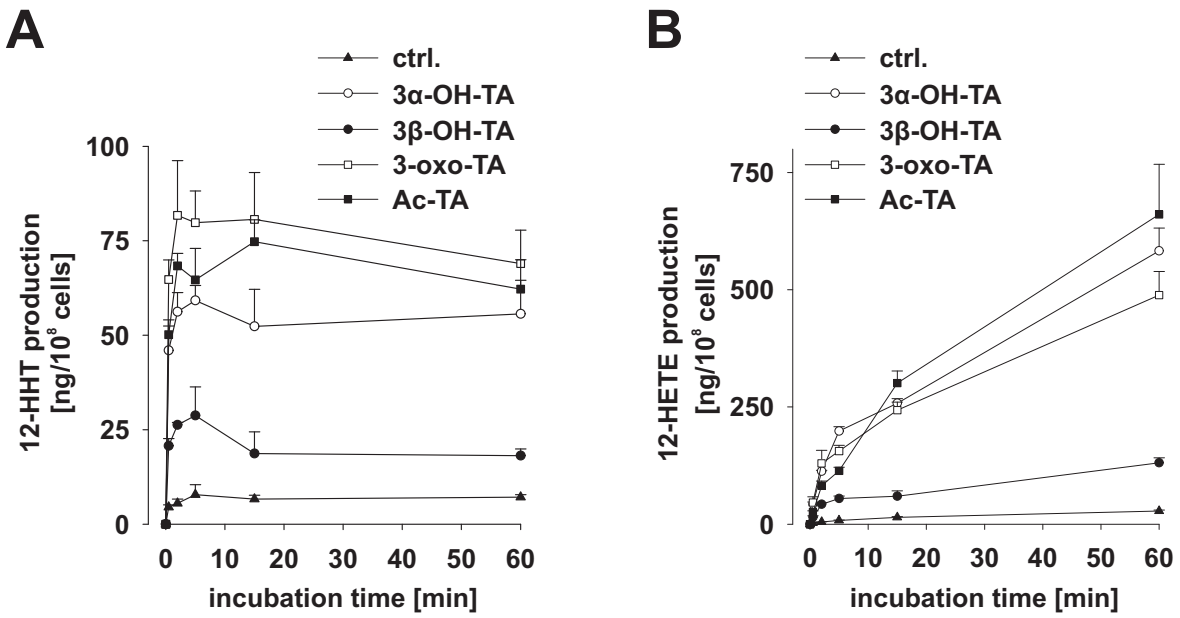


Figure 29: Time-dependency of arachidonic acid metabolite production after treatment with tirucallic acids in human platelets. Platelets (10^8 /ml in PGC buffer) were pre-warmed for 3 min to 37 °C. The samples were incubated with vehicle (ctrl., DMSO), 3α-OH-TA, 3β-OH-TA, 3-oxo-TA or Ac-TA (10 µM) for 30 sec, 2 min, 5 min, 15 min or 60 min at 37 °C. The reaction was terminated and 12-HHT (**A**) and 12-HETE formation (**B**) were determined. Data are given as mean + S.E. in ng per 10^8 cells, n = 3.

4.2.4 Effects of the cPLA$_2$α inhibitor RSC-3388 on tirucallic acid-induced arachidonic acid metabolite production in platelets

AA release in platelets is largely dependent on the activity of the cPLA$_2$α, but can also be mediated by alternative mechanisms [580-581]. To assess the role of cPLA$_2$α, platelets were pre-treated with the established selective cPLA$_2$α inhibitor RSC-3388 [235, 237] and then incubated with TAs (10 µM). In fact, the cPLA$_2$α inhibitor (15 µM) effectively blocked TA- and A23187-induced AA metabolite production to the level of the respective vehicle control (Figure **30 A-D**). Interestingly, in Ca^{2+}-containing buffer (Figure **30 A**) and to a minor extent in Ca^{2+}-deprived samples (Figure **30 B**), absolute 12-HHT production was generally increased after treatment with

the cPLA$_2$α inhibitor. This completely unexpected effect was less prominent for 12-HETE production (Figure **30 C** and **D**).

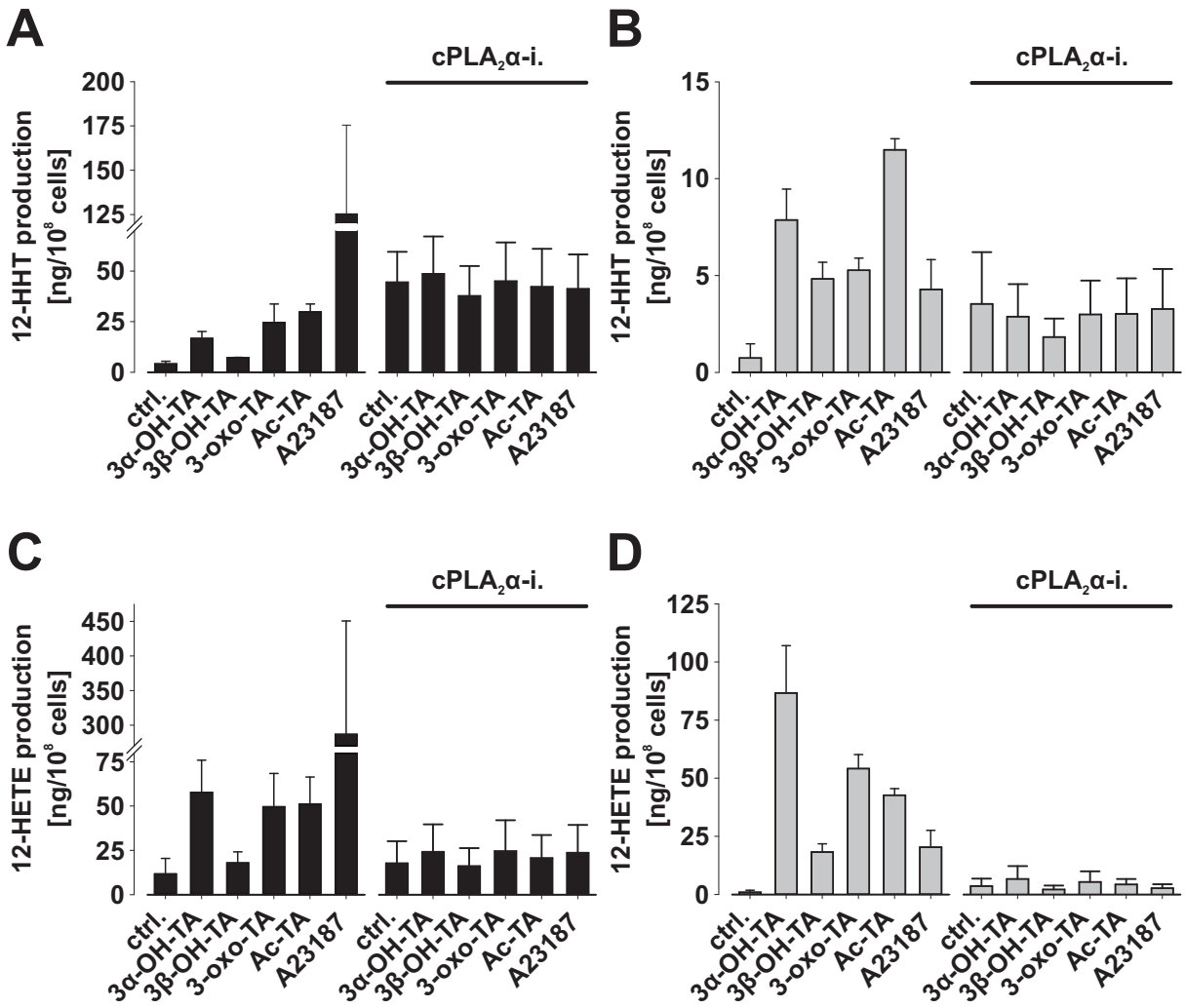


Figure 30: Effect of cPLA$_2$α inhibition on tirucallic acid-induced arachidonic acid metabolite production in human platelets. Platelets (10^8 /ml in PG buffer) were supplemented with 1 mM $CaCl_2$ (1 min, 37 °C, panels **A** and **C**) or pre-treated with 1 mM EDTA and 30 µM BAPTA-AM for 15 min at RT and pre-warmed to 37 °C for 1 min (**B** and **D**). The samples were incubated with vehicle (DMSO) or cPLA$_2$α inhibitor RSC-3388 (cPLA$_2$α-i., 15 µM) for further 15 min at 37 °C and then treated with vehicle (ctrl., DMSO), 3α-OH-TA, 3β-OH-TA, 3-oxo-TA or Ac-TA (10 µM) or A23187 (1 µM) at 37 °C for 5 min. The reaction was terminated and 12-HHT (**A** and **B**) and 12-HETE formation (**C** and **D**) were determined. Data are given as mean + S.E. in ng per 10^8 cells. n = 3.

4.2.5 Subcellular distribution of cPLA$_2$α in platelets after treatment with tirucallic acids

As translocation of the cPLA$_2$α to the platelets' membranes is the initial step leading to cPLA$_2$α activity [135, 582] (see chapters 2.2.2.3 and 2.2.2.4), cPLA$_2$α subcellular localisation was investigated by Western blot after treatment with TAs and subsequent subcellular fractionation in soluble and membranous portions. In Ca^{2+}-containing buffer, treatment of platelets with thrombin (1 U/ml) provoked cPLA$_2$α translocation from the cytosolic to the membranous fraction. In contrast, thrombin failed to induce translocation in the Ca^{2+}-deprived approach (Figure **31**). Although the relative occurrence of cPLA$_2$α in the respective fractions after treatment with the different TAs was fluctuating in individual experiments, TAs clearly led to cPLA$_2$α membrane translocation in Ca^{2+}-containing buffer, with 3-oxo-TA and Ac-TA inducing the most potent effects. In contrast to the thrombin control, the TAs still effectively induced translocation in Ca^{2+}-deprived platelets (Figure **31**, right panel) and the degree of membrane translocation paralleled the induction of eicosanoid formation, with 3α-OH-TA and Ac-TA being most efficient and 3β-OH-TA as weakest inducer (compare Figure **27** and Figure **28** and Table **5**). Especially in 3α-OH-TA-treated samples, translocation seemed to be more distinct at low Ca^{2+} levels.

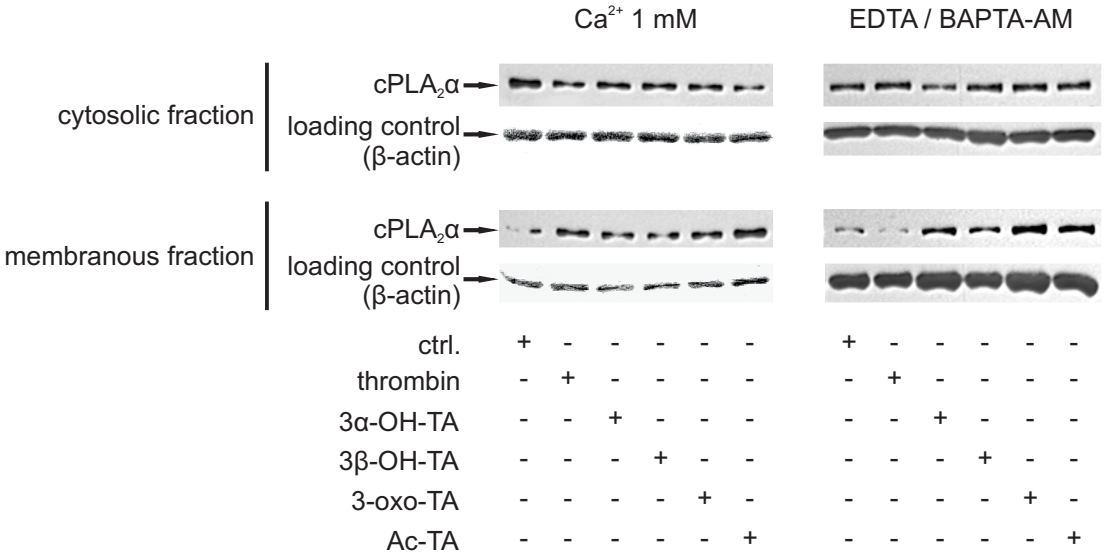


Figure 31: cPLA$_2$α translocation in human platelets after treatment with tirucallic acids. Washed human platelets (5×10^9 /ml) in translocation buffer (134 mM NaCl, 15 mM Tris-HCl pH 7.6, 1 g/l glucose) were either pre-treated with 1 mM EDTA and 30 µM BAPTA-AM (30 min prior to incubation, RT) or 1 mM $CaCl_2$ (5 min prior to incubation). Then, the samples were pre-warmed to 37 °C for 4 min and the test compounds (10 µM), thrombin (1 U/ml) or vehicle (ctrl., DMSO) were added. After 5 min, the incubation was stopped on ice. Protease inhibitors (10 µg/ml leupeptin, 60 µg/ml STI and 1 mM PMSF) were added and the cells were lysed by freeze-thaw cycles and centrifuged at 100,000 × g (45 min, 4 °C). The supernatant (cytosolic fraction) was collected and the pellet (membranous fraction) was resuspended in translocation buffer with protease inhibitors. Both fractions were subjected to SDS-PAGE and Western blot analysis on cPLA$_2$α and β-actin. Results are representative for four experiments.

4.2.6 Impact of tirucallic acids on cPLA$_2$α-driven arachidonic acid release in cell-free models

In order to evaluate potential direct interactions of the TAs and cPLA$_2$α or membrane structures, cell-free assays on AA release from different artificial vesicles were performed. Since cPLA$_2$α activity varies depending on the nature of the substrate and of course depending on the Ca^{2+} levels (Figure **32**), TAs were tested in several setups using different compositions of membranous lipids in Ca^{2+}-containing or Ca^{2+}-deprived buffer. Vesicles composed of 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC) alone (Figure **33 A**) or in combination with 1-palmitoyl-2-oleoyl-*sn*-glycerol (POG, in a ratio of 2:1 n/n, Figure **33 B**) or cholesterol (in ratios of 1:1 n/n, Figure **33 C** or 4:1 n/n, Figure **33 D**), or vesicles composed of 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine (PAPE, Figure **33 E**) were used to obtain membranes with diverse characteristics in terms of rigidity, steric accessibility, and polarity or charge of the surface.

Regardless of the presence of Ca^{2+}, in none of the membrane models TAs appreciably intensified cPLA$_2$α-induced AA release. In mixed vesicles from PAPC and POG and in PAPE vesicles, AA release was rather suppressed with increasing TA concentrations. TAs were also tested in the above-mentioned membranous models using 1 mM EDTA in place of Ca^{2+} (data not shown). The cPLA$_2$α activity in the absence of Ca^{2+} was quite faint in some of the membrane models (see Figure **32**); however, no increase in enzymatic activity was observed after treatment with TAs at concentrations from 3 to 30 µM. Thus, these data might exclude a direct stimulation of cPLA$_2$α by TAs as possible mechanism underlying the stimulatory effects on cellular AA release.

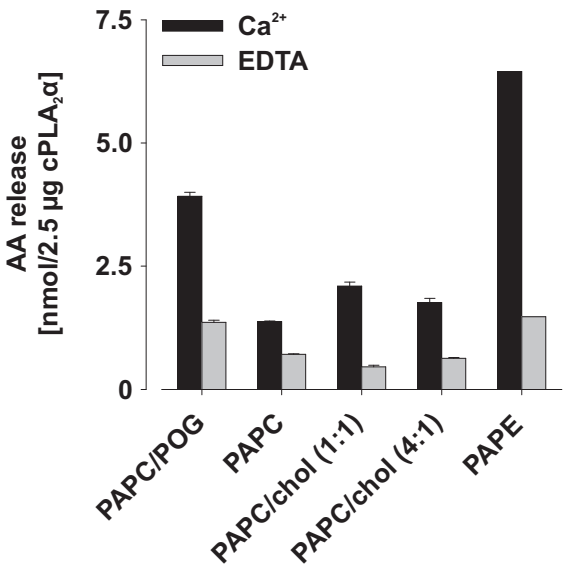


Figure 32: AA release by cPLA$_2$α from various lipid vesicles. Vesicles composed of PAPC alone or in combination with POG (in a ratio of 2:1 n/n) or cholesterol (in ratios of 1:1 n/n or 4:1 n/n), or vesicles composed of PAPE (lipid concentration 250 µM in TBS buffer with 1 mg/ml FAF-BSA) were supplemented with $CaCl_2$ (1 mM) or EDTA (1 mM) and pre-incubated with vehicle (ctrl., DMSO) for 10 min at RT. The reaction was started by addition of the purified cPLA$_2$α enzyme (2.5 µg/ml) and maintained at 37 °C for 60 min. After derivatization, AA was analyzed by HPLC. Data are given as mean + S.E. of the absolute release of AA in nmol per 2.5 µg cPLA$_2$α, n = 1 – 5.

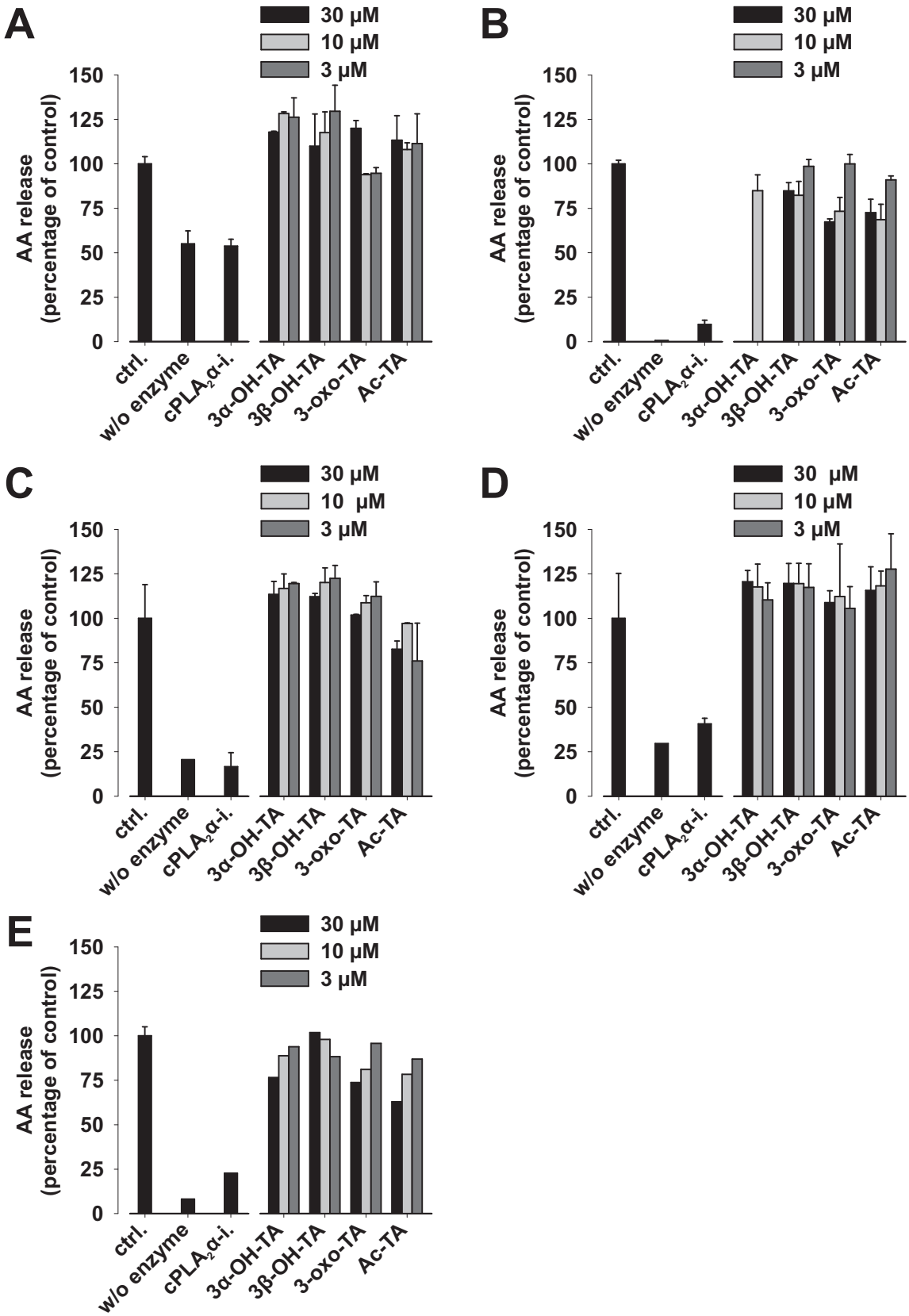


Figure 33: Effects of tirucallic acid on cPLA$_2$α-induced AA release from lipid vesicles. Vesicles composed of PAPC alone (**A**) or in combination with POG (in a ratio of 2:1 n/n, **B**) or cholesterol (in ratios of 1:1 n/n, **C** or 4:1 n/n, **D**) or vesicles composed of PAPE (**E**) (lipid concentration 250 µM in TBS buffer with 1 mg/ml FAF-BSA) were supplemented with $CaCl_2$ (1 mM) and pre-incubated with vehicle (ctrl., DMSO), 5 µM cPLA$_2$α inhibitor (cPLA$_2$α-i.) and 3α-OH-TA, 3β-OH-TA, 3-oxo-TA or Ac-TA at the indicated concentration for 10 min at RT. The reaction was started by addition of the purified cPLA$_2$α enzyme (2.5 µg/ml, 37 °C, 60 min). After derivatization, AA was analyzed by HPLC. Data are given as mean + S.E. of the percentage of the vehicle control, n = 1 – 5.

4.2.7 Calcium mobilization in platelets after treatment with tirucallic acids

Increase of the intracellular Ca^{2+} level is the major trigger for $cPLA_2\alpha$ translocation to membranes [156]. To analyze, whether TA-induced $cPLA_2\alpha$ translocation in Ca^{2+}-containing buffer results from elevated intracellular Ca^{2+} levels, platelets were labelled with Fura-2, a Ca^{2+}-sensitive dye, and Ca^{2+} levels were measured after treatment with TAs. Indeed, TAs conspicuously mediated Ca^{2+} mobilization in platelets (Figure **34**). Compared to the vehicle control, 3-oxo-TA most potently elevated Ca^{2+} levels by about 150 nM within 30 sec; 3α-OH-TA, 3β-OH-TA and Ac-TA led to an increase of 70 nM, 35 nM and 100 nM, respectively (Figure **34 E**). Nevertheless, the effect of the TAs on Ca^{2+} mobilization was much slighter than after stimulation with thrombin (0.5 U/ml, increase of 580 ± 53 nM), thapsigargin (0.1 µM, increase of 440 ± 158 nM) or A23187 (1 µM, increase of 1090 ± 140 nM) but superior to the stimulatory effects of PAF (0.1 µM, increase of 90 ± 4 nM) or collagen (8 µg/ml, increase of 55 ± 8 nM). Ca^{2+} levels were not only transiently increased by the TAs but persisted on a plateau for a period of at least two minutes (Figure **34 A**-**D**). This time response mostly resembled to that of thrombin.

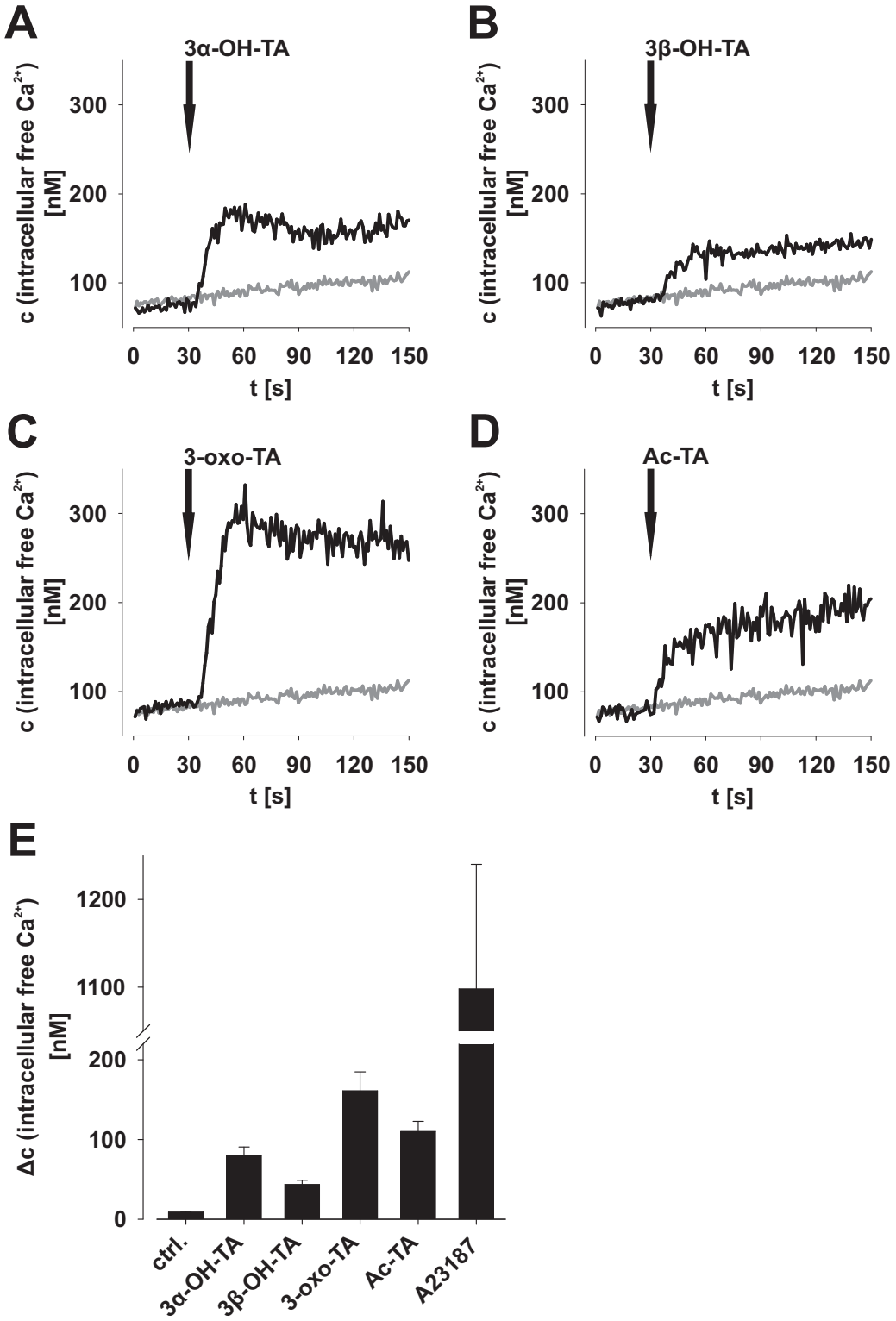


Figure 34: Ca^{2+} mobilization in human platelets by tirucallic acids. Washed Fura-2-AM-labelled washed platelets (10^8 /ml in PG buffer) were incubated in a thermally controlled and constantly stirred fluorometer cuvette (37 °C) in a fluorospectrometer. $CaCl_2$ (1 mM) was added 1 min prior to stimulation with vehicle (ctrl., DMSO, **A**-**E**), 3α-OH-TA (10 µM, **A**, **E**), 3β-OH-TA (10 µM, **B**, **E**), 3-oxo-TA (10 µM, **C**, **E**), Ac-TA (10 µM, **D**, **E**) or A23187 (1 µM, **E**). Fluorescence emission at 510 nm was measured after excitation at 340 nm and 380 nm and intracellular Ca^{2+} was calculated as described. **A-D:** Ca^{2+} levels after treatment with vehicle are illustrated in grey; black curves represent Ca^{2+} levels after stimulation with TAs. Results are representative for three to four experiments. **E:** Δc represents the increase in intracellular free Ca^{2+} before and 30 sec after treatment with TAs, n = 3 – 4.

4.2.8 Effects of tirucallic acids on p42/44, p38 and JNK MAPK and Akt signalling

$cPLA_2\alpha$ activity is intensely regulated by phosphorylation [173, 178-179]. Amongst others, the phosphorylation of Ser^{505} by JNKs (p54/46) [176-177], p42/p44 (ERK-1/-2) [174] and p38 MAPK [583] modulates $cPLA_2\alpha$ enzyme activity. It appeared reasonable to speculate that TAs could induce $cPLA_2\alpha$ translocation and thus AA release by inducing $cPLA_2\alpha$ phosphorylation. To analyze a potential impact of TAs on phosphorylation events, platelets were treated with TAs (10 µM) and then processed by SDS-PAGE and Western blot for analysis of phosphorylated JNK (p46/54), p42/44 and p38 MAPK. In contrast to thrombin, which intensively induced p42/p44 phosphorylation in Ca^{2+}-containing buffer, none of the TAs led to ERK activation within 90 sec (Figure **35**) or 5 min (data not shown). Deprivation of Ca^{2+} did not increase the effect of the TAs but abrogated thrombin-induced ERK phosphorylation. In contrast, p38 MAPK was activated after treatment with thrombin as well as with TAs. Especially Ac-TA induced p38 phosphorylation, but also 3-oxo-TA and 3β-OH-TA led to an activation of this kinase. Phosphorylation of JNK paralleled the activation of p38 but the effect was markedly less obvious. After treatment with EDTA and BAPTA-AM, p38 phosphorylation was generally increased – also in the vehicle-treated samples. 3α-OH-TA and Ac-TA marginally enhanced p38 phosphorylation, whereas an effect of the other TAs or thrombin was barely apparent due to the elevated basal phosphorylation state of the kinase. In the absence of Ca^{2+}, no changes in JNK phosphorylation were perceptible after incubation with thrombin or TAs within 90 sec.

Recently, interference of TAs with Akt-dependent cell proliferation was demonstrated in cancer cells [557]. Furthermore, BAs were shown to activate Akt in platelets [541]. To investigate whether Akt signalling is involved in effects of TAs in platelets, phosphorylation of Akt was analyzed by Western blot. Akt was not phosphorylated in quiescent platelets, neither in Ca^{2+}-containing buffer, nor in Ca^{2+}-deprived cells. Thrombin intensively induced Akt phosphorylation in Ca^{2+}-enriched environment, whereas TAs did not affect Akt phosphorylation. In Ca^{2+}-deprived cells, Akt was not activated, neither by TAs nor by thrombin.

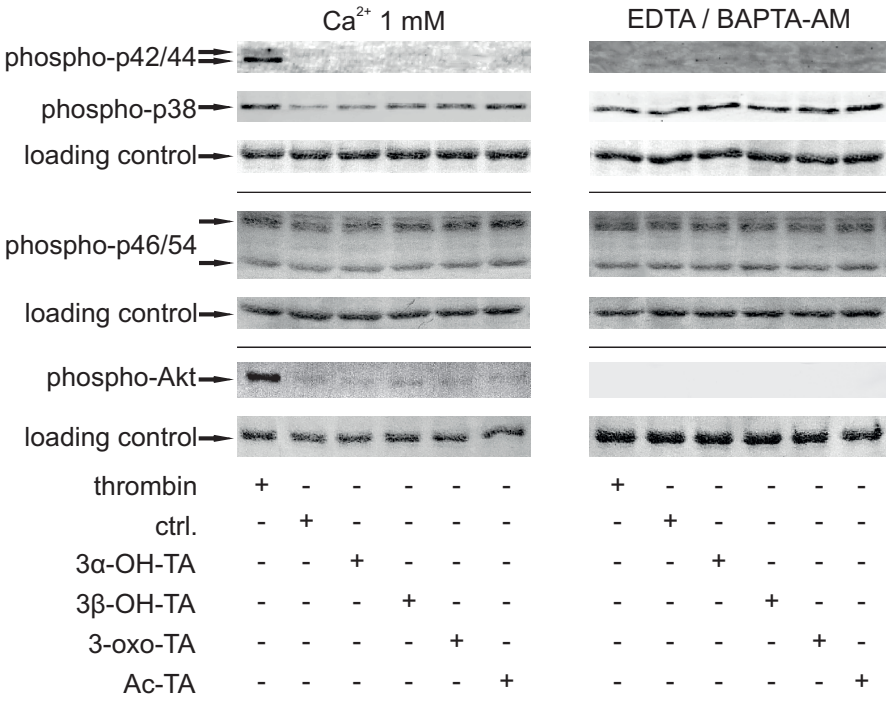


Figure 35: Effect of tirucallic acids on p42/p44 (ERK), p38, p46/54 (JNK) MAPK and Akt phosphorylation in human platelets. Washed human platelets (10^9 /ml) in PG buffer were either pre-treated with 1 mM EDTA and 30 µM BAPTA-AM (15 min prior to incubation, RT) or 1 mM $CaCl_2$ (3 min prior to incubation). The samples were pre-warmed to 37 °C for 3 min and the test compounds (10 µM), thrombin (1 U/ml) or vehicle (ctrl., DMSO) were added. After 5 min in case of Akt and 90 sec in case of the other kinases, the incubation was stopped by addition of SDS loading buffer. The samples were subjected to SDS-PAGE and Western blot analysis on phospho-p42/p44 (ERK), phospho-p38 and phospho-p46/54 (JNK) MAPK and phospho-Akt. Ponceau S-stained protein bands (β-actin) were used as loading control. Results are representative for three or four experiments.

4.2.9 Phosphorylation of cPLA$_2$α at Ser^{505} after treatment of platelets with tirucallic acids

Activated MAPK lead to phosphorylation of cPLA$_2$α at Ser^{505}, which was also investigated in TA-treated platelets by Western blot analysis. Unfortunately, stimulus-induced phosphorylation was only faintly perceivable. Nevertheless, in Ca^{2+}-containing buffer, phosphorylation of cPLA$_2$α was mainly induced after treatment with 3-oxo-TA and Ac-TA (Figure **36**). After deprivation of Ca^{2+}, also the other TAs led to phosphorylation at Ser^{505}.

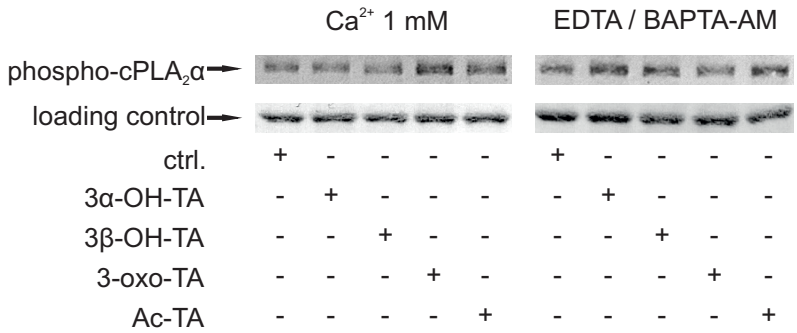


Figure 36: Phosphorylation of cPLA$_2$α (Ser^{505}) after treatment of human platelets with tirucallic acids. Washed human platelets (10^9 /ml) in PG buffer were either pre-treated with 1 mM EDTA and 30 µM BAPTA-AM (15 min prior to incubation, RT) or 1 mM $CaCl_2$ (3 min prior to incubation). The samples were pre-warmed to 37 °C for 3 min and the test compounds (10 µM) or vehicle (ctrl., DMSO) were added. After 5 min, the incubation was stopped by addition of SDS loading buffer. The samples were subjected to SDS-PAGE and Western blot analysis on Ser^{505}-phosphorylated cPLA$_2$α. Ponceau S-stained protein bands (β-actin) were used as loading control. Results are representative for three experiments.

4.2.10 Effects of kinase inhibitors on tirucallic acid-induced arachidonic acid release and metabolite production

For further evaluation of the impact of TAs on cPLA$_2$α-phosphorylating kinases, platelets in Ca^{2+}-containing (Figure **37 A** and **B**) or Ca^{2+}-depleted buffer (Figure **37 C** and **D**) were firstly treated with vehicle, p38 MAPK inhibitor SB203580 (10 µM), p42/p44 MAPK inhibitor U0126 (3 µM), and CaMKII inhibitors KN-62 (5 µM) and KN-93 (10 µM). After 15 min at 37 °C, cells were incubated with vehicle, TAs (10 µM) or thrombin (1 U/ml) for 5 min and 12-HHT (data not shown) and 12-HETE formation were determined. The effects on 12-HHT formation essentially paralleled those on 12-HETE production. Since the kinase inhibitors themselves modulated the metabolite production (Figure **37 A** and **C**), the results were referred to the respective kinase inhibitor-treated controls (Figure **37 B** and **D**). U0126 rather stimulated TA- and thrombin-induced 12-HETE production; only in the absence of Ca^{2+}, 12-HHT (but not 12-HETE) formation was

slightly suppressed by the ERK inhibitor. Of interest, both CaMKII inhibitors significantly suppressed TA-induced 12-HHT and 12-HETE production, in Ca^{2+}-supplemented as well as in Ca^{2+}-depleted cells. SB203580 potently inhibited TA-induced 12-HHT and 12-HETE production in EDTA/BAPTA-AM-treated platelets (Figure **37 D**) but less strikingly repressed their formation in the presence of Ca^{2+} (Figure **37 B**). Interestingly, thrombin-stimulated 12-HETE formation was increased by the p38- and ERK-inhibitors in the presence or absence of Ca^{2+}. In contrast, both CaMKII-inhibitors suppressed thrombin-induced 12-HETE production, especially when Ca^{2+} was present.

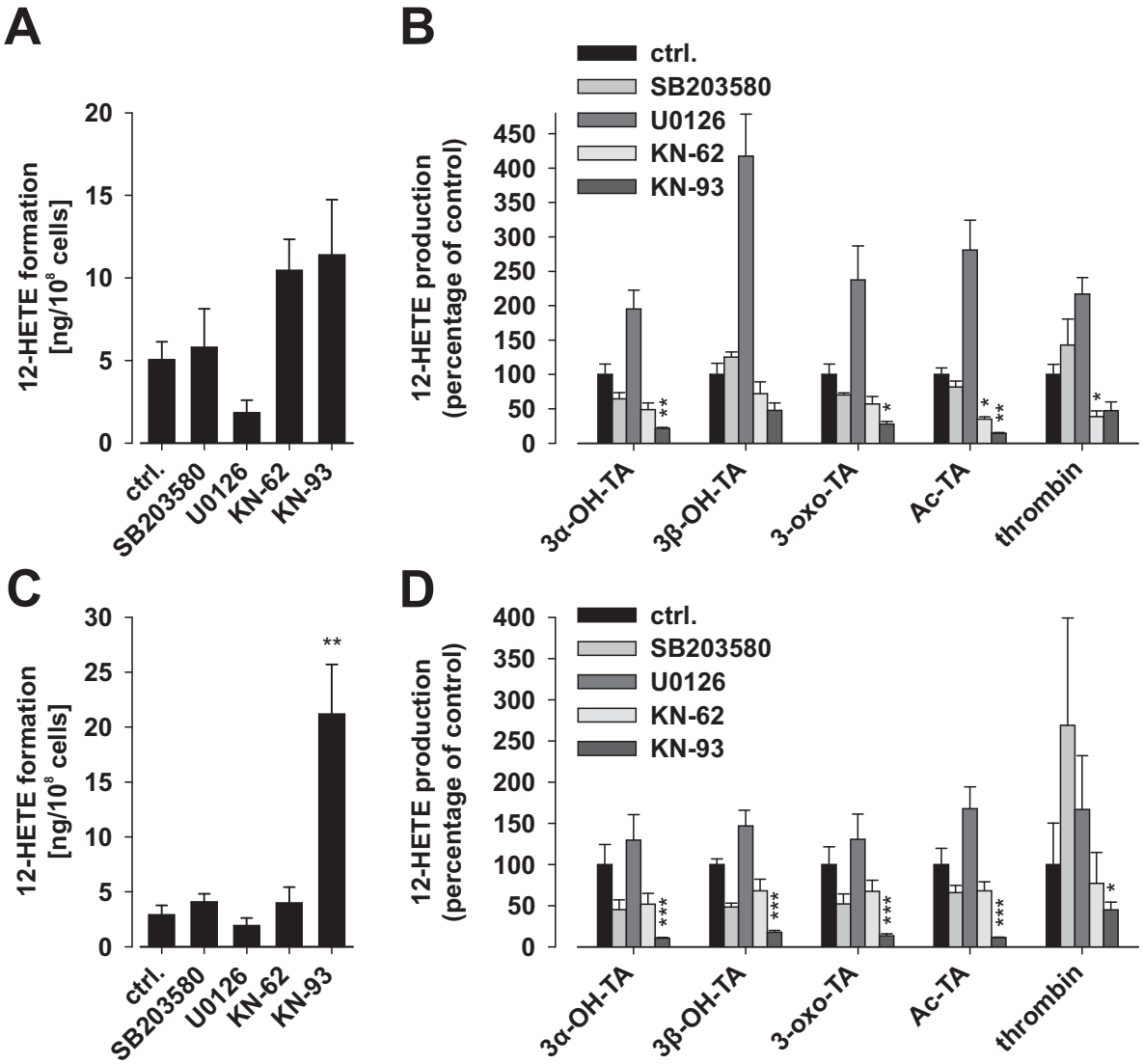


Figure 37: Effects of kinase inhibitors on tirucallic acid-induced 12-HETE formation in human platelets. Platelets (10^8 /ml in PG buffer) were supplemented with 1 mM $CaCl_2$ (1 min, 37 °C, panels **A** and **B**) or treated with 1 mM EDTA and 30 µM BAPTA-AM (15 min prior to incubation, RT, panels **C** and **D**) and incubated with vehicle (ctrl., DMSO), 10 µM SB203580, 3 µM U0126, 5 µM KN-62 or 10 µM KN-93 for 15 min at 37 °C. The samples were treated with vehicle (ctrl., DMSO), 3α-OH-TA, 3β-OH-TA, 3-oxo-TA, Ac-TA (10 µM) or thrombin (1 U/ml) for 5 min at 37 °C. The reaction was terminated and 12-HETE formation was determined. Data are given as mean + S.E. in ng per 10^8 cells (basal 12-HETE formation of the kinase inhibitor-treated vehicle controls, **A** and **C**) or as percentage of the respective kinase inhibitor-treated control (**B** and **D**). n = 3 – 5, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the respective ctrl., one-way ANOVA followed by Tukey-Kramer *post hoc* test.

In addition, TA-induced AA release in platelets was analyzed after pre-treatment with the kinase inhibitors SB203580, KN-93 and KN-62. Unfortunately, the effects of the inhibitors were barely perceptible, which was due to the generally indistinct stimulatory effects of the TAs in these experiments (Figure **38**). 3α-OH-TA most prominently induced AA release and thus, rudimentary suppression of 3α-OH-TA-mediated AA release by KN-93 was the only marked effect that could be noticed. SB203580 did not visibly suppress TA-induced AA release.

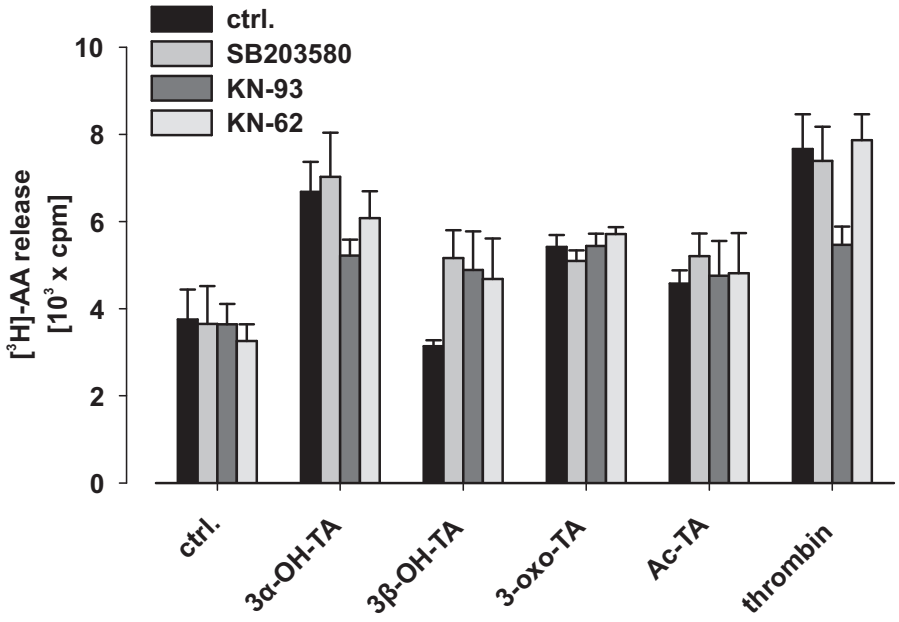


Figure 38: Effect of kinase inhibitors on tirucallic acid-induced arachidonic acid release from human platelets. [^{3}H]-AA-labelled platelets (10^8 /ml in PG buffer) were supplemented with 1 mM $CaCl_2$ (1 min, 37 °C) and incubated with vehicle (ctrl., DMSO), 10 µM SB203580, 10 µM KN-93 or 5 µM KN-62 for 15 min at 37 °C. The samples were stimulated with vehicle (ctrl., DMSO), 3α-OH-TA, 3β-OH-TA, 3-oxo-TA or Ac-TA (10 µM) or thrombin (1 U/ml) at 37 °C for 5 min. The incubation was stopped on ice and cells were spun down. Aliquots of the supernatants were mixed with scintillation cocktail and measured on a scintillation counter to detect released [^{3}H]-AA. Data are given as mean + S.E. in cpm, n = 3.

4.3 Interaction of triterpenic acids and frankincense extracts with PGE_2 biosynthesis

Recently, BAs were identified as inhibitors of mPGES-1 in cell-free, cellular and *in vivo* studies [584]. This chapter evaluates the potency of different extracts from *Boswellia* species and single compounds isolated thereof on mPGES-1-driven PGE_2 synthesis. Furthermore, the impact of the most potent mPGES-1 inhibitors on $cPLA_2\alpha$ and COX isoforms is investigated to assess additive effects leading to reduced PGE_2 levels.

4.3.1 Effects of triterpenic acids and extracts from different *Boswellia* species on mPGES-1 activity in a cell-free system

Extracts from the resin of different *Boswellia* species were analyzed in a cell-free mPGES-1 assay. For this purpose, the resins were extracted with lipophilic solvents (raw extract) and the acid compounds were separated (acid fraction, a. f.) from the neutral compounds (neutral fraction, n. f.). MK-886 (10 µM), possessing an IC_{50} value of 2 µM in a cell-free mPGES-1 activity assay [585], was used as reference compound and led to about 80% inhibition of human mPGES-1 activity. The residual PGE_2 formation was not suppressed by elevated concentrations of MK-886, indicating alternative routes mediating this basal PGE_2 formation. The acid fractions of all the four tested species potently inhibited the enzyme activity at a concentration of 10 µg/ml (Figure **39 A**). In contrast, the neutral fraction from *B. carteri* extract did not impair mPGES-1 activity. As expected, also the raw extracts from *B. serrata* and *B. papyrifera* were less potent than their respective acid fractions (data not shown). Concentration response analysis revealed IC_{50} values of 1.9, 2.8, 1.6 and 0.42 µg/ml for the acid fractions of *B. serrata*, *B. sacra*, *B. carteri* and *B. papyrifera*, respectively (Figure **39 B**). Particularly the acid fraction of *B. papyrifera* potently inhibited the enzyme activity with a maximal inhibition of 92% at 30 µg/ml, which was superior to the control inhibitor MK-886.

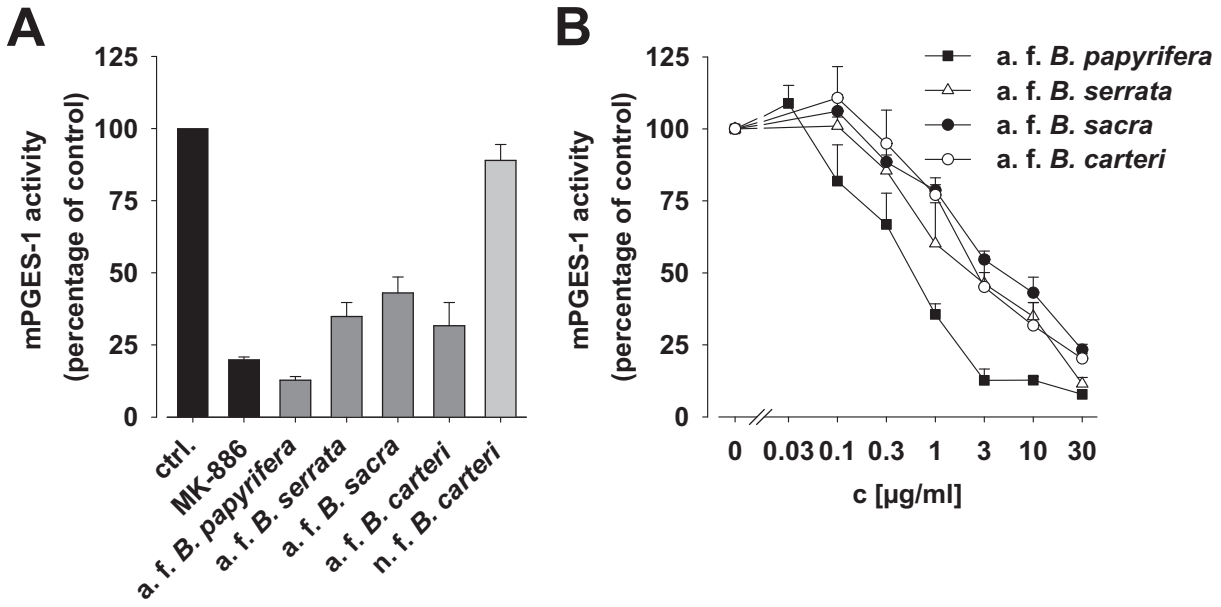


Figure 39: Effects of fractions derived from extracts of *Boswellia* spec. oleo-gum resin on mPGES-1 activity. Microsomal preparations of IL-1β-stimulated A549 cells were pre-incubated with vehicle (ctrl., DMSO), MK-886 (10 µM), acid fractions (a. f.) or neutral fractions (n. f.) from extracts of B. *papyrifera*, *B. serrata*, *B. sacra* or *B. carteri* oleo-gum resins (10 µg/ml in panel **A** and at the respective concentrations in panel **B** for 15 min at 4 °C. The reaction was started by addition of 20 µM PGH_2. After 60 sec at 4 °C, the reaction was terminated using a stop solution containing $FeCl_2$. Data are given as mean + SEM, n = 3 – 4.

The acid fraction of *B. papyrifera* was separated into subfractions by flash chromatography to identify the active principles of the extract. The subfractions were analyzed by normal silica phase thin layer chromatography and fractions with identical components were combined. This separation led to seven fractions, which were in turn tested in the cell-free mPGES-1 assay. All fractions showed inhibitory potential with 65 to 87% inhibition at 3 µg/ml. "Fraction 4" was the most potent fraction, inhibiting the enzyme nearly completely at this concentration. Thus, this fraction was further separated via preparative HPLC and the isolated single substances were analyzed by MS and NMR analytics. "Fraction 4" was found to contain 3-oxo-TA as main component along with 3α-Ac-8,24-dien-TA, β-BA, Ac-LA and A-BA as minor components.

Since further triterpenic acids beyond BAs appear to be potent inhibitors of mPGES-1 activity, a variety of such terpenes isolated from *Boswellia* species were screened on their impact on mPGES-1 activity, including different TAs, RAs and LAs (Figure **40**). At 10 µM, all TAs, DH-k-RA and Ac-OH-LA markedly inhibited PGE_2 production. LA and Ac-LA were only moderately active and RA, DH-RA and DH-NA were nearly ineffective in this assay.

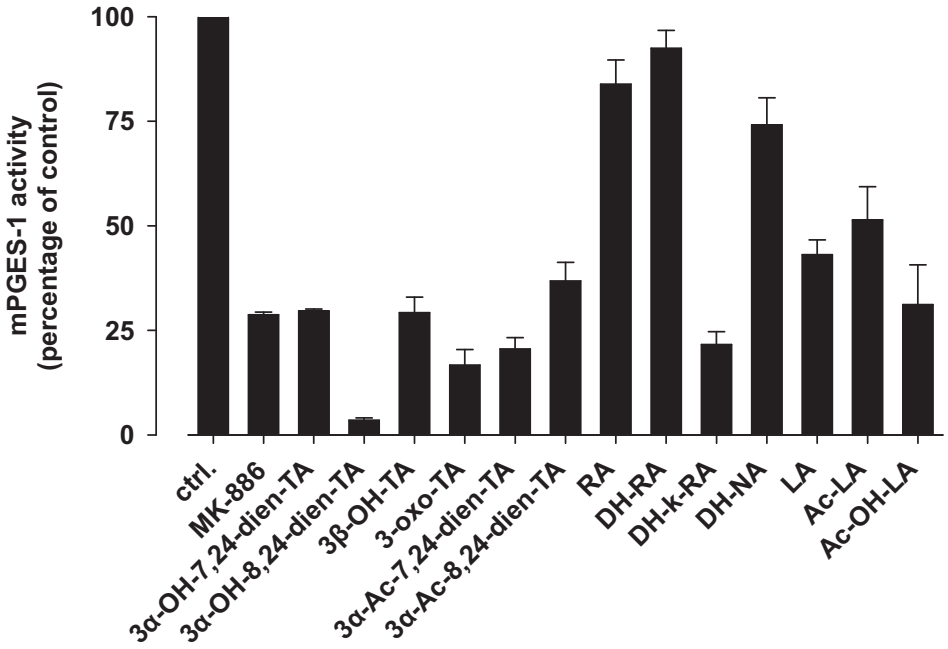


Figure 40: Impact of triterpenic acids from *Boswellia* spec. on mPGES-1 activity. Microsomal preparations of IL-1β-stimulated A549 cells were pre-incubated with vehicle (ctrl., DMSO), MK-886 (10 µM) and the respective triterpenic acids (10 µM) for 15 min at 4 °C. The reaction was started by addition of 20 µM PGH_2. After 60 sec at 4 °C, the reaction was terminated using a stop solution containing $FeCl_2$. Data are given as mean + SEM, n = 3 – 6.

The triterpenic acids exhibiting more than 60% inhibition at 10 µM were subjected to concentration-response analysis (Figure **41 A**-**H**). The tested compounds, namely 3α-OH-7,24-dien-TA, 3α-OH-8,24-dien-TA, 3β-OH-TA, 3-oxo-TA, 3α-Ac-7,24-dien-TA, 3α-Ac-8,24-dien-TA, DH-k-RA and Ac-OH-LA show IC_{50} values of 0.4 to 3 µM (Table **1**). Hence, these compounds are up to eightfold more active in this assay compared to the most potent BA β-BA. Just like MK-886, all compounds exerted a maximal inhibition of about 70 to 80%. Interestingly, only 3α-OH-8,24-TA was able to suppress PGE_2 formation to about 96% (Figure **41 B**).

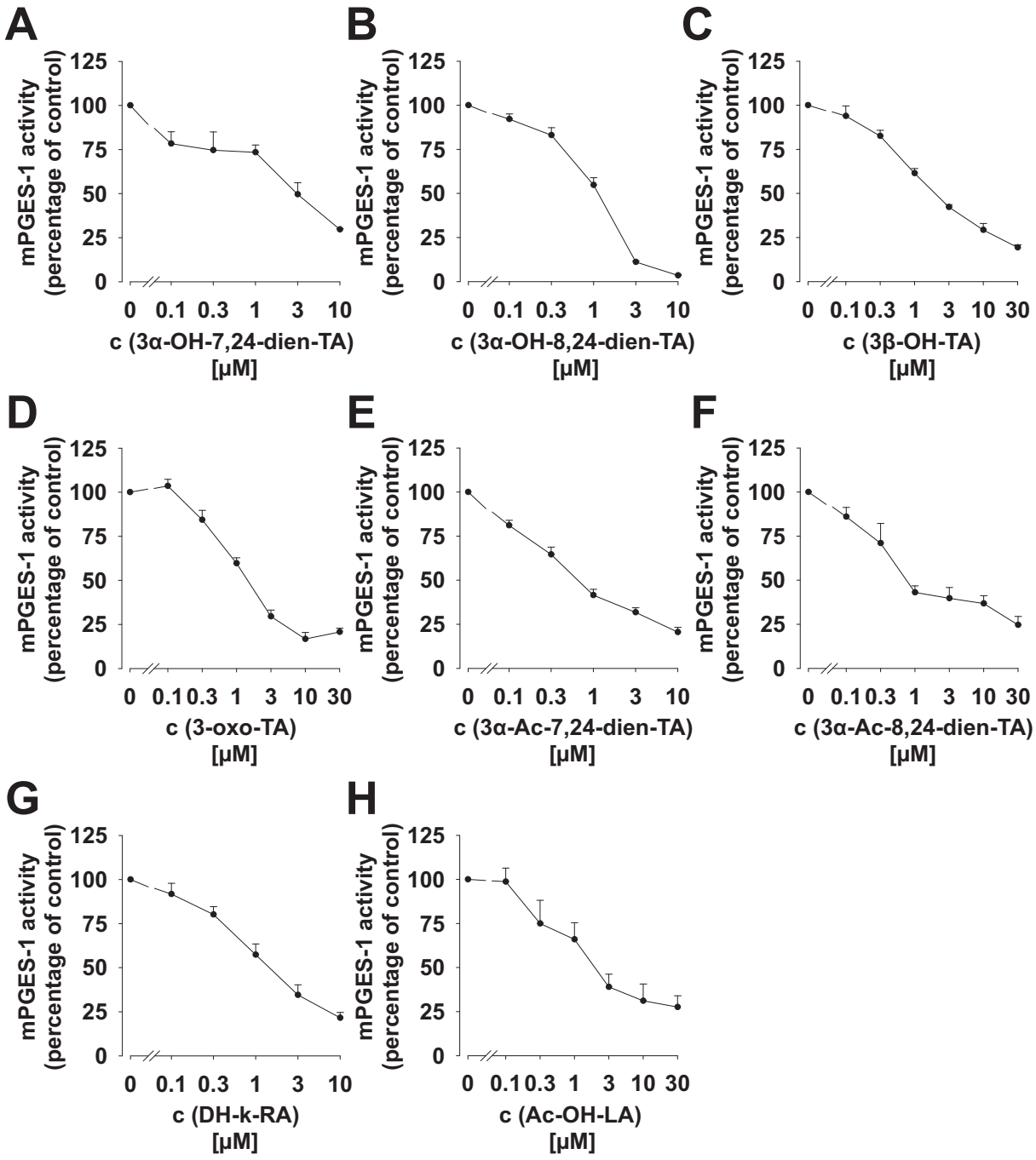


Figure 41: Concentration-response analysis for triterpenic acids from *Boswellia* spec. on mPGES-1 activity. Microsomal preparations of IL-1β-stimulated A549 cells were pre-incubated with 3α-OH-7,24-dien-TA (**A**), 3α-OH-8,24-dien-TA (**B**), 3β-OH-TA (**C**), 3-oxo-TA (**D**), 3α-Ac-7,24-dien-TA (**E**), 3α-Ac-8,24-dien-TA (**F**), DH-k-RA (**G**) or Ac-OH-LA (**H**) at the indicated concentrations for 15 min at 4 °C. The reaction was started by addition of 20 µM PGH_2. After 60 sec at 4 °C the reaction was terminated using a stop solution containing $FeCl_2$. Data are given as mean + SEM, n = 3 – 8.

Table 6: IC_{50} values of triterpenic acids on mPGES-1 activity in a cell-free model. Microsomal preparations of IL-1β-stimulated A549 cells were pre-incubated with the indicated compounds at concentrations of 0.1 to 30 µM for 15 min at 4 °C. The reaction was started by addition of 20 µM PGH_2. After 60 sec at 4 °C, the reaction was terminated using a stop solution containing $FeCl_2$. IC_{50} values were determined by fitting concentration response data to a four parameter logistic curve.

compound	IC_{50} [µM]	compound	IC_{50} [µM]	compound	IC_{50} [µM]
3α-OH-7,24-dien-TA	3.0	3-oxo-TA	0.9	DH-k-RA	1.0
3α-OH-8,24-dien-TA	1.1	3α-Ac-7,24-dien-TA	0.4	Ac-OH-LA	0.9
3β-OH-TA	1.2	3α-Ac-8,24-dien-TA	0.4		

3-oxo-TA, the major compound in the most active fraction ("fraction 4") of the extract from *B. papyrifera* (acid fraction) appears to contribute strongly to the inhibitory potential of the crude extract in this cell-free assay. Nevertheless, "fraction 4" and the acid fraction of the extract from *B. papyrifera* themselves were at least equally potent compared to 3-oxo-TA (Figure **42**).

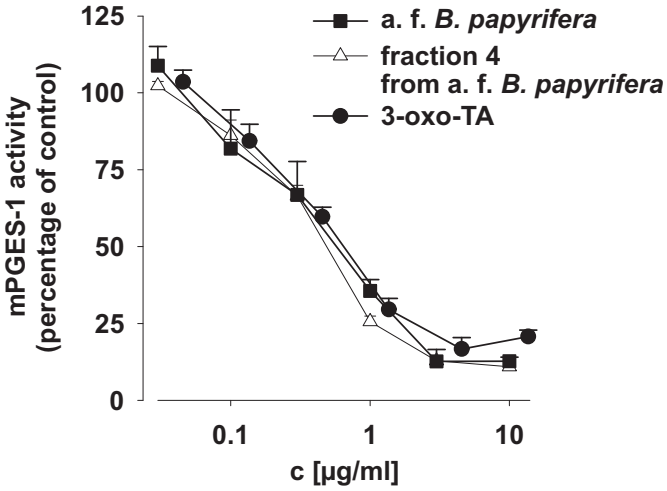


Figure 42: Comparison of the potency of the acid fraction of the extract from *B. papyrifera*, its subfraction "fraction 4" and its major component 3-oxo-TA for mPGES-1 inhibition. Microsomal preparations of IL-1β-stimulated A549 cells were pre-incubated with the acid fraction of the extract from *B. papyrifera* (a. f. *B. papyrifera*), its most active fraction ("fraction 4" from a. f. *B. papyrifera*) and its major component 3-oxo-TA at the indicated concentrations for 15 min at 4 °C. The reaction was started by addition of 20 µM PGH_2. After 60 sec at 4 °C, the reaction was terminated using a stop solution containing $FeCl_2$. Data are given as mean + SEM, n = 3 – 8.

4.3.2 PGE_2 synthesis in LPS-primed and AA-stimulated monocytes after treatment with triterpenic acids or extracts from different *Boswellia* species

To evaluate the inhibition of mPGES-1 in a cellular model, monocytes were isolated from human whole blood and incubated with LPS for 20 h to stimulate the upregulation of mPGES-1. In fact, stimulation with LPS intensively induces mPGES-1 expression in human monocytes, while cPGES and mPGES-2 levels remain unaltered [586]. Subsequently, the monocytes were treated with the test compounds and PGE_2 formation was induced by incubation with AA. Basal PGE_2 formation in monocytes, which were not treated with LPS or AA gave only about 10% of the entirely stimulated vehicle control (LPS + AA) (Figure **43**). Without LPS-treatment, PGE_2 was only sparsely synthesized after stimulation with AA (about 18% of the entirely stimulated vehicle control), representing the PGE_2 that was seemingly produced independently of mPGES-1. After priming with LPS but without stimulation with AA, substantial PGE_2 formation (about 60% compared to the entirely stimulated vehicle control) was detected. This basal PGE_2 may mainly derive from mPGES-1, using endogenous AA provided by cellular phospholipases. The synthetic reference drug MD-52 (2 µM), a selective mPGES-1 inhibitor [587], inhibited PGE_2 formation in stimulated (LPS + AA) monocytes by about 58%. Thus, selective inhibition of mPGES-1 suppresses AA-induced as well as basal PGE_2 formation in this test system. Similarly, the COX inhibitor indomethacin (10 µM) inhibited PGE_2 formation by about 62%. As exogenous AA is a substrate of COX enzymes and only secondarily of mPGES-1, the impact of the test compounds on both enzymes is monitored in this assay.

Some of the triterpenic acids were able to suppress PGE_2 formation in this test system. At 10 µM, 3α-OH-7,24-dien-TA, 3α-OH-8,24-dien-TA and 3β-OH-TA inhibited PGE_2 formation by 20 to 30%, with 3α-OH-8,24-dien-TA being the most potent inhibitor, which totally blocked the AA-induced PGE_2 formation at a concentration of 1 µM. Comparably, 3-oxo-TA effectively suppressed PGE_2 formation at 1 and 3 µM but the inhibitory effect decreased at 10 µM. 3α-Ac-7,24-dien-TA, DH-k-RA and Ac-OH-LA exhibited only little or no effectiveness in this model. Unexpectedly, 3α-Ac-8,24-dien-TA even stimulated PGE_2 synthesis.

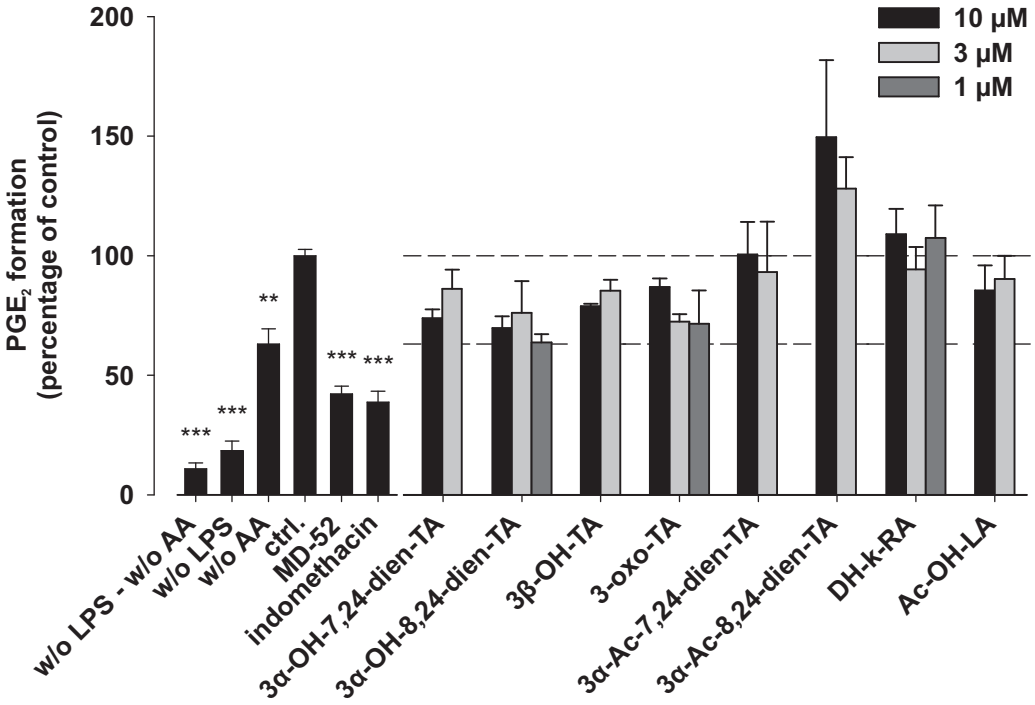


Figure 43: Impact of triterpenic acids on PGE_2 formation in LPS-primed AA-stimulated monocytes. Human monocytes (10^6 /ml in 1 ml RPMI medium supplemented with FCS (0.5%, v/v), penicillin (100 U/ml) and streptomycin (100 µg/ml)) were primed with LPS (1 µg/ml, 20 h, 37 °C, 6% CO_2, except "w/o LPS" samples) and incubated with vehicle (ctrl., DMSO), indomethacin (10 µM), MD-52 (2 µM) and the respective triterpenic acids at the indicated concentrations for 15 min (37 °C, 6% CO_2) prior to addition of stimuli to induce PGE_2 formation. The reaction was started by addition of 1 µM AA (except "w/o AA" samples). After 30 min, the medium was collected, centrifuged and the PGE_2 content in the supernatant was determined by ELISA. Data are given as mean + S.E. of the percentage of the vehicle control with 1,606 ± 182 pg PGE_2 per 10^6 cells. n = 3 – 7, ** $p < 0.005$, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey-Kramer *post hoc* test.

4.3.3 Effects of triterpenic acids and extracts from *Boswellia* species on PGE_2 and 6-keto $PGF_{1\alpha}$ synthesis in LPS-stimulated whole blood

The triterpenic acids were tested in a human whole blood model to evaluate their potency in a more physiological context, using an appropriate test system for differential assessment of COX-2/mPGES-1-derived PGE_2 [585]. Therefore, human whole blood was buffered with potassium phosphate buffer and treated with thromboxane synthase inhibitor CV-4151. After treatment with the test compounds for 10 min at RT the samples were incubated with LPS (10 µg/ml) for 5 h at 37 °C. The content of PGE_2 and 6-keto $PGF_{1\alpha}$ in the plasma was quantified by ELISA. The parallel determination of 6-keto $PGF_{1\alpha}$ allows the assessment of inhibitory effects that appear upstream of PGE_2 synthesis itself, e.g. by inhibition of COX. Samples that were not stimulated with LPS produced about 35% of PGE_2 compared to the stimulated vehicle control (Figure **44**). Treatment with indomethacin (10 µM) led to PGE_2 levels that were even lower as the non-stimulated control. The selective mPGES-1 inhibitor MD-52 (6 µM) only suppressed PGE_2 formation by about 33%, which may represent the maximal inhibition that can be achieved by

exclusive inhibition of mPGES-1 in this model. The triterpenic acids (10 µM) also partially inhibited PGE_2 formation (Figure **44 A**), with 3β-OH-TA, 3-oxo-TA, DH-k-RA and Ac-OH-LA being as potent as MD-52. The Ac-TAs and 3α-OH-TAs were less effective or totally failed to suppress PGE_2 formation. 6-keto $PGF_{1\alpha}$ production was significantly suppressed by indomethacin (leading to about 15% 6-keto $PGF_{1\alpha}$ vs. the stimulated vehicle control), but it was not affected by any of the test compounds (data not shown). This implies that the test compounds inhibit PGE_2 formation by acting on mPGES-1 rather than on COX or PLA_2 enzymes.

Acid fractions of extracts from *Boswellia* species oleo-gum resin were tested in this whole blood assay as well (Figure **44 B**). At 3 µg/ml, the acid fraction from *B. papyrifera* and *B. serrata* inhibited PGE_2 formation comparably to MD-52. The extract a. f. *B. sacra* was less effective and a. f. *B. carteri* did not inhibit PGE_2 formation. Interestingly, at higher concentrations, inhibition of PGE_2 formation by a. f. *B. papyrifera* was less distinct (data not shown).

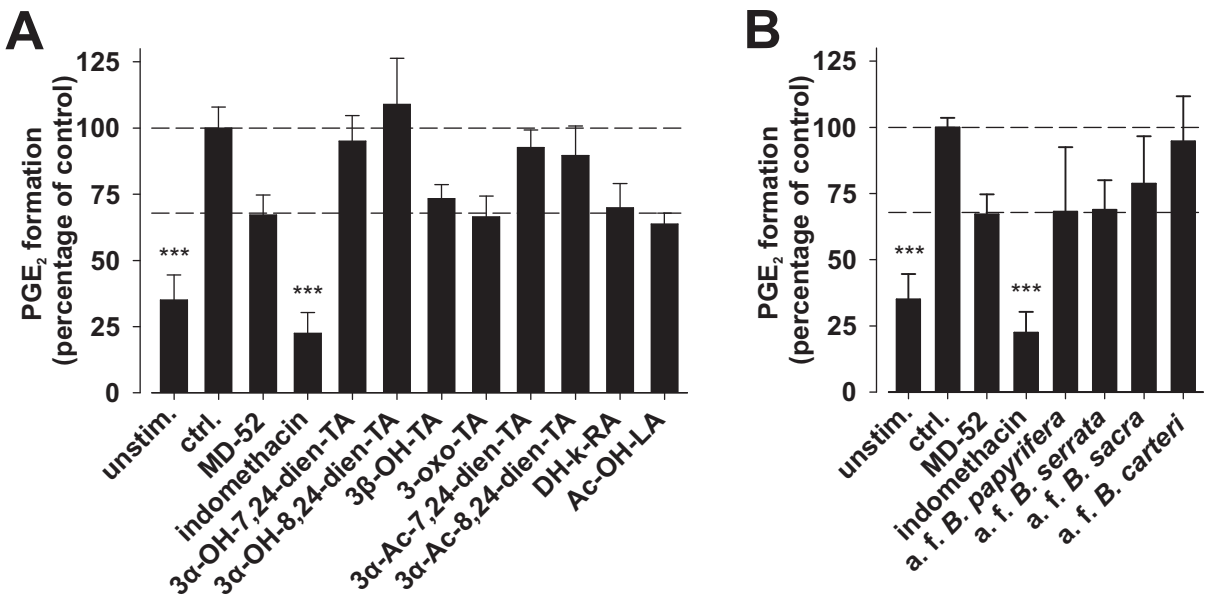


Figure 44: Impact of triterpenic acids and extracts from *Boswellia* species oleo-gum resins on PGE_2 formation in LPS-stimulated human whole blood. Heparinized human whole blood was buffered with phosphate buffer (20%, v/v), treated with CV-4151 (1 µM) and incubated with vehicle (ctrl., DMSO), MD-52 (6 µM), indomethacin (10 µM) and the respective triterpenic acids (10 µM, panel **A**) or the acid fractions (a. f.) of extracts from *Boswellia* species oleo-gum resins (3 µg/ml, panel **B**) for 10 min at RT. The reaction was started by addition of 10 µg/ml LPS (except "unstim." sample). After 5 h at 37 °C, the reaction was stopped on ice. The samples were centrifuged, the supernatants acidified with citric acid and centrifuged again. The supernatant was processed by SPE and HPLC, the PGE_2 peaks were collected and the PGE_2 content was determined by ELISA. Data are given as mean + S.E. of the percentage of the vehicle control with 3,836 ± 549 pg PGE_2 per ml blood. n = 4 – 6, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey-Kramer *post hoc* test.

4.3.4 Impact of triterpenic acids and extracts from *Boswellia* species on the activity of COX-1 and -2

To estimate the selectivity of the triterpenic acids, which performed the most potent inhibition of mPGES-1 activity, the impact on further enzymes participating in PGE_2 synthesis was analyzed. In cell-free assays using purified ovine COX-1 (Figure **45 A**) and purified recombinant human COX-2 (Figure **45 C**), the triterpenic acids elicited inhibitory effects but these were much less intense than those found for the inhibition of mPGES-1. Significant inhibition was only accomplished by some of the tested compounds at very high concentrations (100 µM), namely by 3-oxo-TA and Ac-OH-LA in COX-1 and COX-2 assays and DH-k-RA in the COX-2 assay. Interestingly, also the reference drug indomethacin (10 µM) only suppressed COX-1 activity to about 40%. Obviously, inhibition of 12-HHT formation beyond this level is barely feasible in this assay.

In addition, acid and neutral fractions of extracts from the *Boswellia* species oleo-gum resins were tested (10 µg/ml) for their impact on COX-1 and -2 activity. The acid fractions - foremost those of *B. papyrifera* and *B. carteri* - exerted moderate inhibition of COX-1 (Figure **45 B**), whereas the neutral fraction from *B. carteri* was ineffective. COX-2 activity was slightly suppressed by the acid fractions, with the acid fractions from *B. serrata* and *B. sacra* leading to significant inhibition of the isolated enzyme at 10 µg/ml (Figure **45 D**).

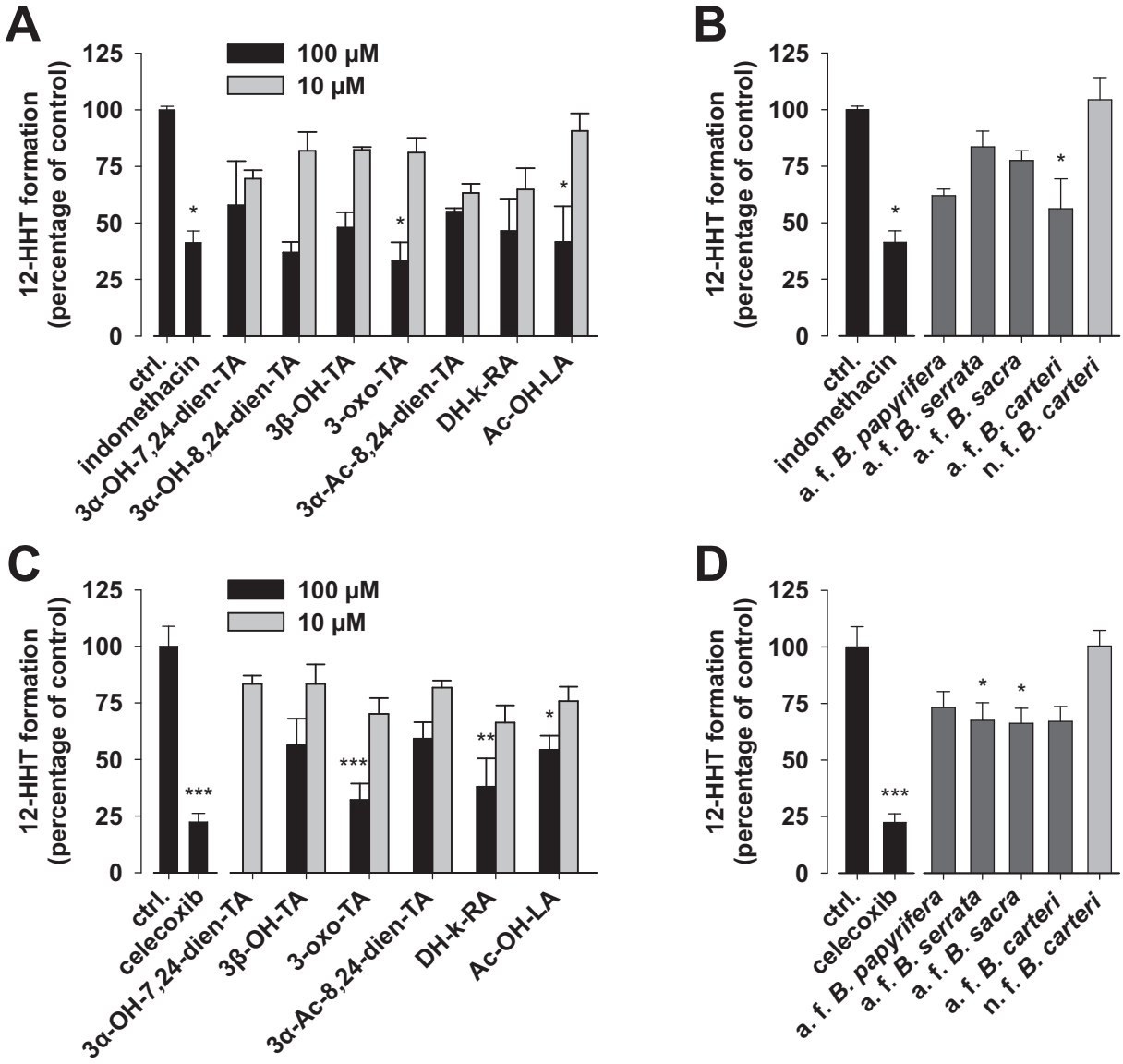


Figure 45: Impact of triterpenic acids and extracts from *Boswellia* species on cyclooxygenase-1 and -2 activity in cell-free assays. Purified COX-1 (ovine, 50 units, panels **A** and **B**) or COX-2 (human recombinant, 20 units, panels **C** and **D**) in Tris buffer (100 mM, pH 8) supplemented with 5 mM glutathione, 5 µM haemoglobin and 100 µM EDTA were incubated with vehicle (ctrl., DMSO), indomethacin (10 µM) or celecoxib (10 µM), the indicated test compounds at concentrations of 100 µM and 10 µM (panels **A** and **C**) or the acid (a. f.) or neutral fraction (n. f.) of extracts from the indicated *Boswellia* species at 10 µg/ml (panels **B** and **D**) for 9 min at 4 °C. Samples were pre-warmed at 37 °C for 1 min and AA (5 µM for COX-1, 2 µM for COX-2) was added. After 5 min at 37 °C, the reaction was stopped and 12-HHT formation was determined. Data are given as mean + S.E. of the percentage of the vehicle control with 443 ± 46.8 ng 12-HHT per 50 units COX-1 (**A** and **B**) and 220 ± 64.5 ng 12-HHT per 20 units COX-2 (**C** and **D**). n = 3 – 6, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey-Kramer *post hoc* test.

As the impact of COX inhibitors in cell-free approaches frequently deviates from the potency in cellular systems, the effects of the triterpenic acids were additionally analyzed in cell-based assays on COX activity. COX-1 activity was tested in human platelets after stimulation with AA (5 µM). In contrast to the cell-free model, indomethacin (10 µM) almost totally suppressed COX-1 activity here (Figure **46**). The triterpenic acids also suppressed COX-1 activity, but only DH-k-RA led to more than 50% inhibition at 10 µM.

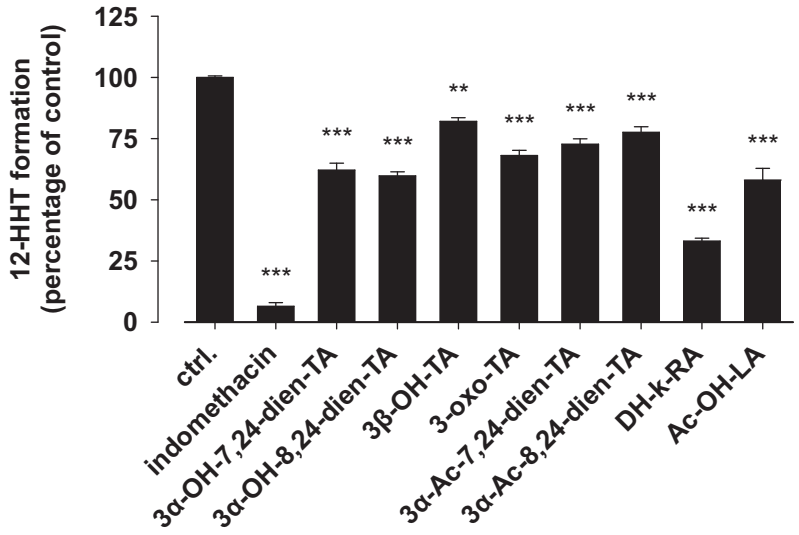


Figure 46: Effects of triterpenic acids on COX-1-dependent 12-HHT formation in human platelets. Platelets (10^8/ml in PGC buffer) were pre-incubated with vehicle (ctrl., DMSO), indomethacin (10 µM) and the respective triterpenic acids (10 µM) for 4 min at RT and 1 min at 37 °C. The cells were stimulated with AA 5 µM for 5 min at 37 °C. The reaction was terminated and 12-HHT formation was determined. Data are given as mean + S.E. of the percentage of the vehicle control with 163 ± 10.5 ng 12-HHT per 10^8 cells, n = 4, ** $p < 0.01$, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey HSD *post hoc* test.

Cellular COX-2 activity was tested in IL-1β-primed, AA-stimulated A549 cells. Priming of A549 cells with IL-1β leads to upregulation of COX-2 and mPGES-1 [333]; COX-1 is seemingly not expressed in A549 cells [588]. Thus, 6-keto $PGF_{1\alpha}$ formation in consequence of stimulation with exogenous AA is an appropriate indicator for cellular COX-2 activity. AA-induced 6-keto $PGF_{1\alpha}$ formation was blocked by pre-treatment with indomethacin (Figure **47**). However, none of the analyzed triterpenic acids inhibited COX-2-driven 6-keto $PGF_{1\alpha}$ formation at a concentration of 10 µM.

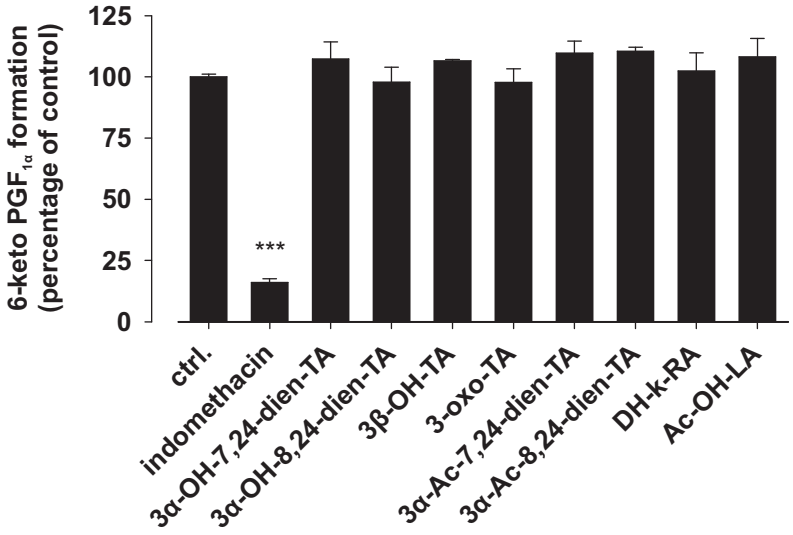


Figure 47: Effects of triterpenic acids on COX-2-dependent 6-keto $PGF_{1\alpha}$ formation in IL-1β-primed, AA-stimulated A549 cells. A549 cells (2×10^6 cells in 20 ml DMEM/high glucose (4.5 g/l) medium containing FCS (10%, v/v), penicillin (100 U/ml) and streptomycin (100 µg/ml)) were incubated for 16 h (37 °C, 6% CO_2). The medium was replaced by fresh medium containing 2% (v/v) of FCS and induction of COX-2 was started by addition of IL-1β (2 ng/ml) for 72 h (37 °C, 6% CO_2). The cells were detached, washed twice with PBS buffer and resuspended (2×10^6 /ml) in PGC buffer. Cells were incubated with vehicle (ctrl., DMSO), indomethacin (20 µM) and the respective triterpenic acids (10 µM) for 10 min (37 °C) and the reaction was started by addition of 3 µM AA and stopped on ice after 15 min (37 °C). The cells were spun down and 6-keto $PGF_{1\alpha}$ in the supernatant was determined using a 6-keto $PGF_{1\alpha}$ ELISA kit. Data are given as mean + S.E. of the percentage of the vehicle control with 386 ± 50.7 pg 6-keto $PGF_{1\alpha}$ per 2×10^6 cells, n = 3, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey HSD *post hoc* test.

The production of COX-derived 12-HHT after treatment with triterpenic acids and stimulation with calcium ionophore A23187 was also tested in human whole blood. Stimulation with A23187 led to about fourfold increase in 12-HHT production that was vastly inhibited by ibuprofen (100 µM) (Figure **48**). Remarkably, the four tested TAs (10 µM) suppressed the A23187-stimulated 12-HHT production to about 60%, though not significantly. DH-k-RA was less effective (20% inhibition) and Ac-OH-LA did not affect or rather stimulated 12-HHT production. Of interest, increasing concentrations of the TAs (30 µM) did not enhance their inhibitory potential on 12-HHT formation (data not shown). It should be noted that other eicosanoids (5-LO products and 12-HETE) were not or only marginally suppressed at a concentration of 10 µM (data not shown).

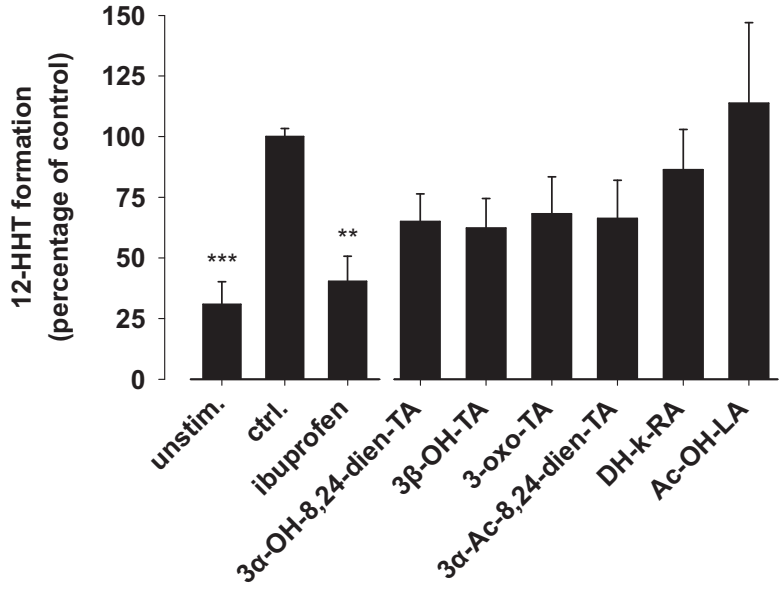


Figure 48: Effects of triterpenic acids on 12-HHT formation in A23187-stimulated human whole blood. Heparinized human whole blood was pre-incubated with vehicle (ctrl., DMSO), ibuprofen 100 µM, and the indicated triterpenic acids 10 µM for 10 min at 37 °C. Samples were stimulated with A23187 (30 µM) for 10 min (37 °C), the incubation was stopped on ice and the content of 12-HHT was determined by HPLC. Data are given as mean + S.E. of the percentage of the vehicle control with 75.5 ± 16.9 ng 12-HHT per ml blood, n = 3 – 6, ** $p < 0.01$, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey-Kramer *post hoc* test.

4.3.5 Impact of triterpenic acids and extracts from *Boswellia* species on $cPLA_2$ activity

The impact of the most potent mPGES-1-inhibiting triterpenic acids on $cPLA_2\alpha$ activity was tested in a cell-free assay, using purified recombinant $cPLA_2\alpha$ as enzyme source and PAPC/POG-vesicles as substrate. AA release in this model was largely suppressed by the synthetic $cPLA_2\alpha$ inhibitor ($cPLA_2\alpha$-i., RSC-3388). The TAs and DH-k-RA only moderately inhibited AA release with a maximal inhibition of about 30% after treatment with 3-oxo-TA or 3α-Ac-8,24-dien-TA (10 and 30 µM) (Figure **49 A**). Obviously, increasing concentrations of these compounds did not lead to enhanced inhibition. On the other hand, Ac-OH-LA potently inhibited the activity of $cPLA_2\alpha$ (see chapter 4.1). The neutral fraction from *B. carteri* did not affect $cPLA_2\alpha$-induced AA release (Figure **49 B**). The acid fractions from *B. sacra* and *B. carteri* were ineffective at this concentration as well. In contrast, the acid fractions from *B. papyrifera* and *B. serrata* significantly suppressed AA release to 54 and 72%, respectively. At higher concentrations (30 µg/ml), the inhibitory effect was not enhanced but remained at the same level (data not shown).

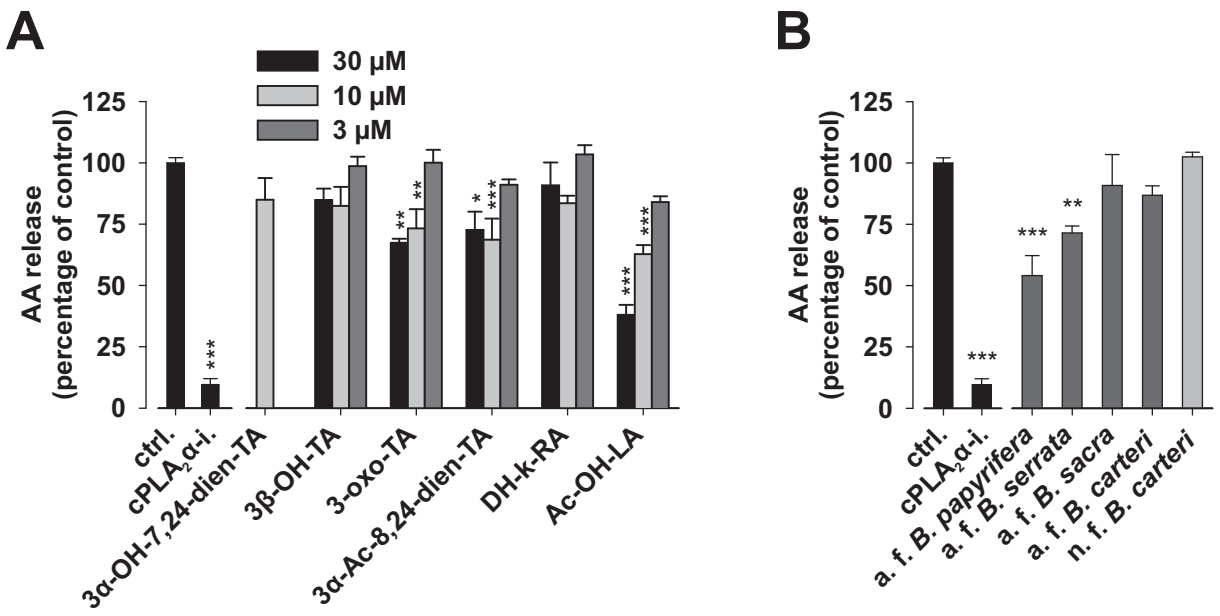


Figure 49: Effects of triterpenic acids and extracts from *Boswellia* species on AA release from PAPC/POG vesicles by purified cPLA$_2$α. PAPC/POG-vesicles (lipid concentration 250 µM in TBS buffer containing 1 mg/ml FAF-BSA) were supplemented with $CaCl_2$ (1 mM) and pre-incubated with vehicle (ctrl., DMSO), 5 µM cPLA$_2$α inhibitor (cPLA$_2$α-i.), triterpenic acids (**A**) at the indicated concentrations or the acid (a. f.) or neutral fraction (n. f.) of extracts from the indicated *Boswellia* species oleo-gum resins at 10 µg/ml (**B**) for 10 min at RT. The reaction was started by addition of the purified enzyme (2.5 µg/ml) and maintained at 37 °C for 60 min. After derivatization, AA was analyzed by HPLC. Data are given as mean + S.E. of the percentage of the vehicle control, n = 3 – 6, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey-Kramer *post hoc* test.

Using [^{3}H]-AA-labelled platelets as cellular test system, the TAs further increased A23187-induced AA release as expected, especially at higher concentrations (see chapter 4.2, Figure **50 A**). As described in chapter 4.1, Ac-OH-LA concentration-dependently suppressed AA release. Interestingly, in contrast to the cell-free assay on cPLA$_2$α activity, DH-k-RA effectively inhibited AA release in this cellular system. Nevertheless, this inhibition was markedly less potent than for Ac-OH-LA in an assay omitting BSA in the buffer (data not shown), where it stagnated on the half-maximal level that was reached by Ac-OH-LA or the cPLA$_2$α control inhibitor. The acid fractions from *B. papyrifera* and *B. serrata* suppressed AA release at concentrations of 3 µg/ml, but suppression vanished when higher concentrations were applied.

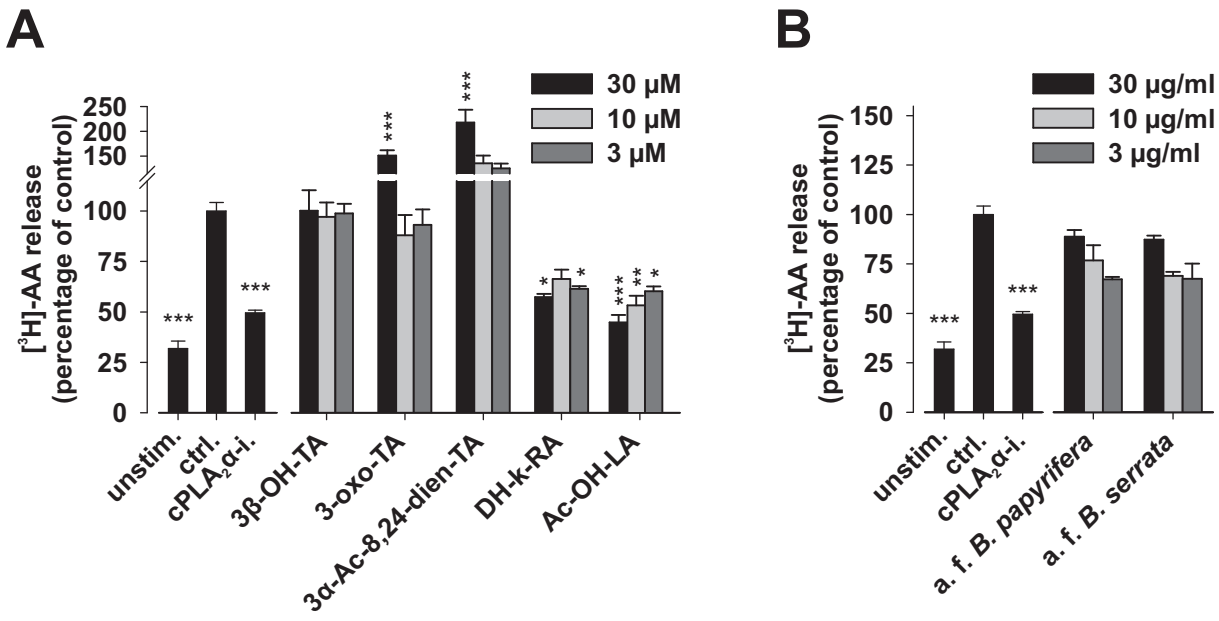


Figure 50: Effects of triterpenic acids and extracts from *Boswellia* species on [^{3}H]-AA release from A23187-stimulated platelets. Labelled platelets (10^8 /ml in PGC buffer supplemented with FAF-BSA 1 mg/ml) were pre-incubated with vehicle (ctrl., DMSO), 5 µM $cPLA_2\alpha$ inhibitor ($cPLA_2\alpha$-i.), triterpenic acids (**A**) or the acid fraction (a. f.) of extracts from the indicated *Boswellia* species at the indicated concentrations (**B**) for 15 min at 37 °C. The cells were stimulated with A23187 1 µM for 5 min at 37 °C. The reaction was terminated on ice, the samples were centrifuged and the supernatant was analyzed by scintillation counting. Data are given as mean + S.E. of the percentage of the vehicle control (+ A23187 1 µM) with $(9.64 \pm 1.36) \times 10^3$ cpm, n = 3 – 6, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey-Kramer *post hoc* test.

4.3.6 Effects of 3-oxo-tirucallic acid and an extract from *B. papyrifera* on carrageenan-induced pleurisy in rats

In order to investigate the inhibition of mPGES-1 *in vivo*, 3-oxo-TA and the acid fraction of the extracted resin from *B. papyrifera* (a. f. *B. papyrifera*) were tested in a carrageenan-induced rat pleurisy model. The extract (a. f. *B. papyrifera*) was selected as it was the most potent extract to inhibit mPGES-1 from all species that were tested. 3-oxo-TA was chosen, as TAs in general turned out to inhibit mPGES-1 activity more or less selectively and 3-oxo-TA represents the major TA in the extract from *B. papyrifera* [498]. The injection of carrageenan in the pleural cavity led to an intense inflammatory process including oedema formation, migration of leukocytes into the pleural space and the production of inflammatory mediators. As biomarkers of the inflammatory reaction, the volume of exudate and the number of infiltrated inflammatory cells in the pleural cavity were determined. PGE_2 was analyzed to assess the impact on PGE_2 synthases and another COX product, 6-keto $PGF_{1\alpha}$, was quantified to discriminate unspecific effects that arise from interference with structures upstream of COX or with COX itself. Production of LTB_4 was determined to monitor effects on 5-LO (Figure **51**). The COX inhibitor indomethacin (5 mg/kg), the FLAP inhibitor MK-886 (1.5 mg/kg) and the direct 5-LO inhibitor zileuton (10 mg/kg) were

used as control drugs. It should be noted that the IC_{50} value of MK-886 for inhibition of FLAP is clearly lower than for mPGES-1 [585, 589] and inhibition of mPGES-1 in not expected in the applied dosage. Vehicle, the control inhibitors, 3-oxo-TA and the extract were administered intraperitoneally 30 min prior to the injection of carrageenan, using DMSO (4% in 1.5 ml saline) as vehicle. Administration of vehicle alone had no effect on carrageenan-induced pleurisy compared to exclusive application of saline (430 ± 34.3 and 487 ± 28.7 µl of gathered exudate, respectively). The COX inhibitor indomethacin reduced the exudate volume to 25%, and only 35% of inflammatory cells infiltrated the pleural cavity. Reduction of PGE_2 and 6-keto $PGF_{1\alpha}$ synthesis to 10 and 6%, respectively, was extremely significant. LTB_4 levels were not significantly reduced to about 84%. The effect of MK-886 (1.5 mg/kg) on the inflammatory symptoms was only sparse. The exudate volume was reduced to 63% and the cell count to 69%. As expected, the LTB_4 levels were significantly suppressed to 25%, whereas the PGE_2 and 6-keto $PGF_{1\alpha}$ levels were not markedly affected. The direct 5-LO inhibitor zileuton (10 mg/kg) clearly diminished the exudate volume to 31% of the control; the cell count was also lowered, but only to 65%. Zileuton not only inhibited LTB_4 production, but also the formation of the COX products 6-keto $PGF_{1\alpha}$ and PGE_2, which was recently investigated in detail [590]. Treatment with 3-oxo-TA (5 mg/kg), though not significantly, reduced the accumulation of exudate to about 78%. The migration of inflammatory cells into the pleural cavity was significantly attenuated to 70%. PGE_2 was significantly reduced to 71%, 6-keto $PGF_{1\alpha}$ and LTB_4 formation dropped to 65 and 77%, respectively. The extract (a. f. *B. papyrifera*, 10 mg/kg) barely affected the exudate volume. On the other hand, it significantly suppressed the number of inflammatory cells to 72%. The synthesis of PGE_2 was suppressed to 79%, thus less potently than after treatment with 3-oxo-TA. 6-keto $PGF_{1\alpha}$ was significantly inhibited to 62%. Comparably to treatment with 3-oxo-TA, LTB_4 declined to 76%. Apparently, both treatments, 3-oxo-TA and the extract, reduced more or less all of the analyzed parameters. Nevertheless, compared to the extract, 3-oxo-TA tended to exhibit more inhibitory activity on PGE_2 formation than on 6-keto $PGF_{1\alpha}$ production. However, the effects on the inflammatory reaction reflected by exudate formation, cell infiltration and the analyzed eicosanoids were less manifest than after treatment with the reference drugs.

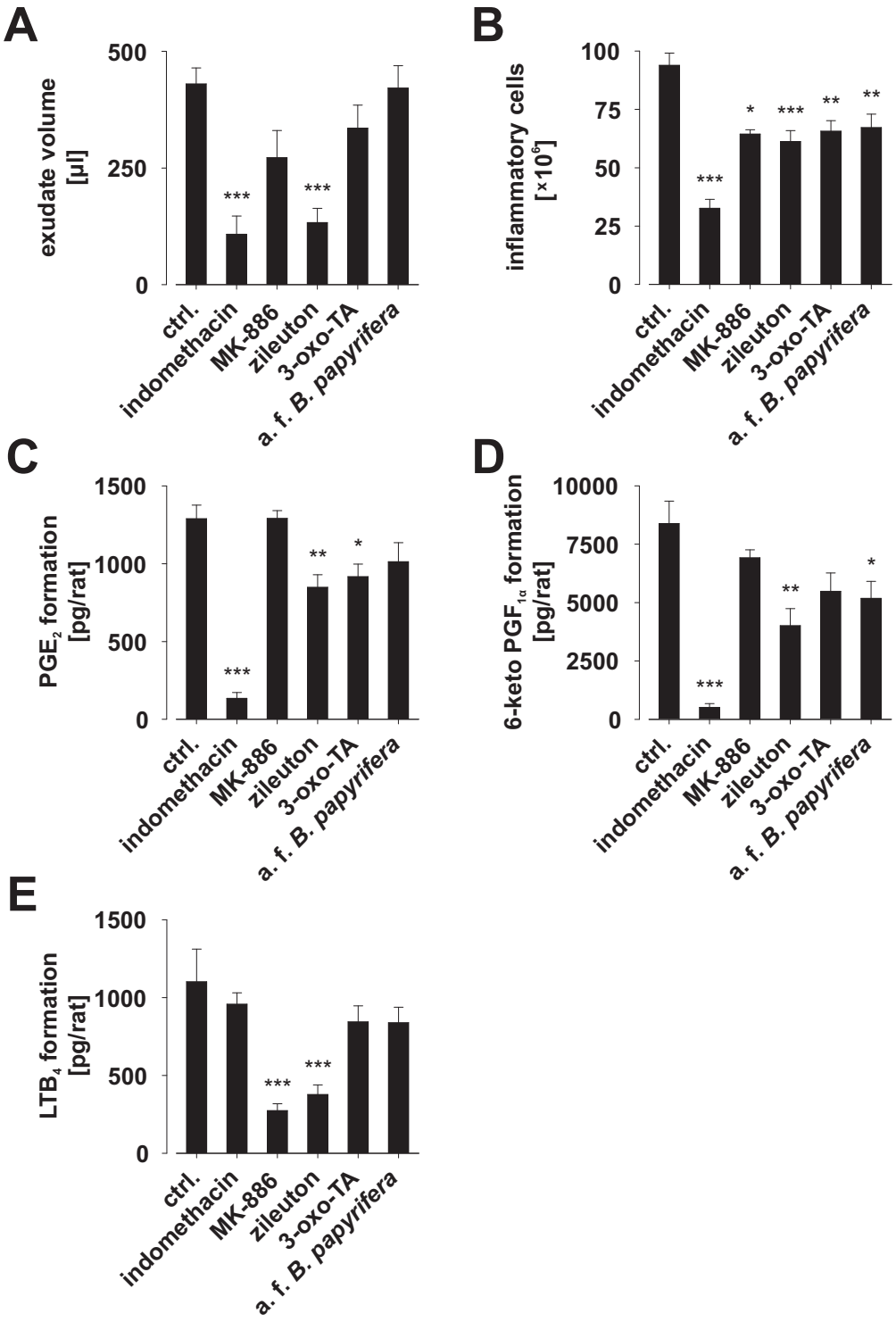


Figure 51: Impact of 3-oxo-TA and of the extract a. f. *B. papyrifera* on carrageenan-induced pleurisy in rats. Indomethacin (5 mg/kg), MK-886 (1.5 mg/kg), zileuton (10 mg/kg), 3-oxo-TA (5 mg/kg) or the acid fraction of the extracted resin from *B. papyrifera* (a. f. *B. papyrifera*, 10 mg/kg) were dissolved in DMSO, diluted in saline (1:25, v/v) and administered intraperitoneally (i. p.) in an overall volume of 1.5 ml 30 min before administration of carrageenan. The vehicle-treated group of rats received 1.5 ml of DMSO 4% (v/v) in saline. Rats were anaesthetized and saline (0.2 ml) or λ-Carrageenan type IV 1% (w/v) (0.2 ml) were injected into the pleural cavity. At 4 h after the injection of λ-carrageenan, the animals were killed and the exudate in the pleural cavity was removed by aspiration. The exudate volume was measured (**A**), leukocytes in the exudate were spun down (800 × g, 10 min) and resuspended in PBS for cell counting (**B**). The amounts of PGE_2 (**C**), 6-keto $PGF_{1\alpha}$ (**D**) and LTB_4 (**E**) in the supernatant of the exudate were determined by radioimmunoassay (PGE_2) or ELISA (LTB_4 and 6-keto $PGF_{1\alpha}$). The results are expressed as mean ± S.E. of 5 – 20 rats in µl per rat (**A**), inflammatory cells per rat (**B**) or pg per rat (**C**, **D**, **E**), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey-Kramer *post hoc* test.

Summing up, TAs, DH-k-RA and Ac-OH-LA potently inhibit mPGES-1 activity in a cell-free test system. The inhibitory potential of 3-OH-TAs and 3-oxo-TA on PGE_2 formation was maintained in LPS-primed monocytes, whereas the Ac-TAs, DH-k-RA and Ac-OH-LA lost their effectiveness in this model. In human whole blood, DH-k-RA, Ac-OH-LA, 3β-OH-TA and 3-oxo-TA potently inhibited PGE_2 formation comparably to the selective mPGES-1 inhibitor MD-52. Assays evaluating the activity of COX-1 and -2 showed only moderate effects of the triterpenic acids at concentrations of 10 µM, though COX-2 was not affected in cellular models. $cPLA_2\alpha$ activity was potently suppressed by Ac-OH-LA, but only little effects were seen after treatment with TAs and DH-k-RA. In a carrageenan-induced pleurisy model in rats, 3-oxo-TA and the extract a. f. *B. papyrifera* significantly reduced the accumulation of inflammatory cells in the pleural cavity. The formation of PGE_2, 6-keto $PGF_{1\alpha}$ and LTB_4 were moderately reduced.

4.4 Impact of triterpenic acids and extracts from *Boswellia* species on 5-LO

5-LO is the first pharmacological target that was identified for BAs [530]. Nonetheless, the contribution of other triterpenic acids than BAs to the inhibition of 5-LO by extracts from *Boswellia* species has been barely considered.

4.4.1 Effects of triterpenic acids and extracts from *Boswellia* species on cell-free 5-LO activity

In a cell-free test system, using semi-purified human recombinant 5-LO, extracts from different *Boswellia* species were tested on their 5-LO-inhibiting potential. The synthetic 5-LO inhibitor BWA4C (0.3 µM) reduced 5-LO product formation to 36% remaining activity. The acid fractions of the extracts (10 µg/ml) considerably suppressed 5-LO product formation to levels of 54 to 67%, whereas the neutral fraction of *B. carteri* extract was clearly less potent (Figure **52**). As the pattern of acids composing extracts from different species differs markedly, the comparable effects of the acid fractions might be due to a similar content of a few active compounds (e.g. BAs) or due to a broad variety of active components that result in similar potency.

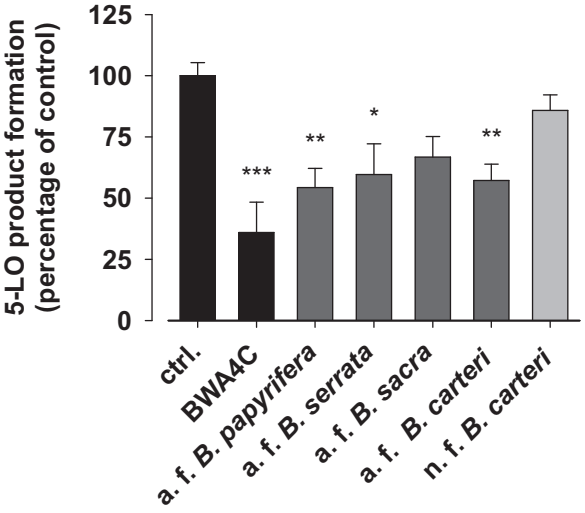


Figure 52: Effects of extracts from *Boswellia* spec. on the activity of purified 5-lipoxygenase. 0.5 µg of purified recombinant 5-LO in cold PBS containing 1 mM EDTA and 1 mM ATP were incubated with vehicle (ctrl., DMSO), 0.3 µM BWA4C or the acid fractions (a. f.) or neutral fraction (n. f.) of extracts from the indicated *Boswellia* species (10 µg/ml) for 10 min (4 °C). The samples were pre-warmed at 37 °C for 30 sec and the reaction was started by addition of 2 mM $CaCl_2$ and 20 µM AA. After 10 min, the reaction was stopped and the 5-LO products 5(S)-HETE and the all-trans isomers of LTB_4 were quantified by HPLC. Data are given as mean + SEM of the percentage of the vehicle control with 1,296 ± 378 ng 5-LO products per 0.5 µg 5-LO, n = 4 – 5. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey-Kramer *post hoc* test.

Next, the efficacy of triterpenic acids from different *Boswellia* species was assessed in the same cell-free system. First, compounds were analyzed at 10 µM and those that caused more than 25% inhibition were tested also at lower concentrations (Figure **53**). The triterpenic acids were compared to the most common BAs (right side of the chart). Representatives of different structural subgroups, especially 3β-OH-TA, 3-oxo-TA, 3α-OH-8,24-dien-TA, DH-k-RA and Ac-OH-LA, significantly inhibited 5-LO activity and their potency was comparable to that of the BAs.

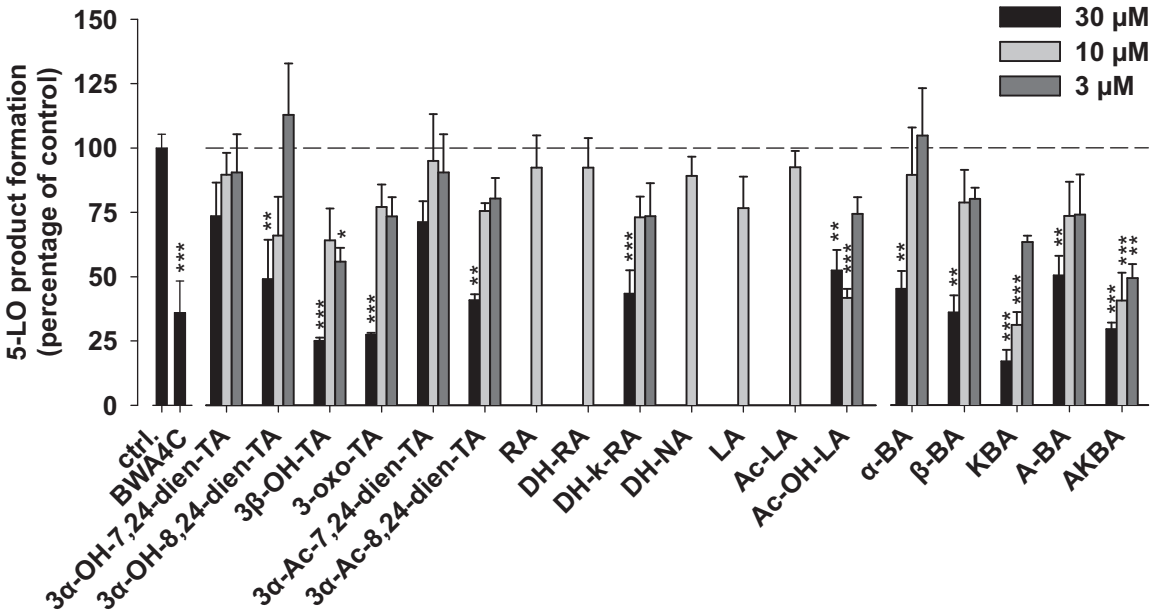


Figure 53: Impact of triterpenic acids from *Boswellia* spec. on isolated 5-lipoxygenase. 0.5 µg of purified recombinant 5-LO in cold PBS containing 1 mM EDTA and 1 mM ATP were incubated with vehicle (ctrl., DMSO), 0.3 µM BWA4C or the indicated compounds for 10 min (4 °C). The samples were pre-warmed at 37 °C for 30 sec and the reaction was started by addition of 2 mM $CaCl_2$ and 20 µM AA. After 10 min the reaction was stopped and the 5-LO products 5(S)-HETE and the all-trans isomers of LTB_4 were quantified by HPLC. Data are given as mean + SEM of the percentage of the vehicle control with 1,401 ± 120 ng 5-LO products per 0.5 µg 5-LO, n = 3 – 5. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey-Kramer *post hoc* test.

4.4.2 Effects of triterpenic acids from *Boswellia* species on 5-LO product formation in stimulated human neutrophils

To evaluate the effects of triterpenic acids on 5-LO in a cellular environment, PMNL from human whole blood were treated with the compounds (10 µM) and then stimulated with 2.5 µM A23187 and 20 µM AA. This stimulation allows a relatively selective conclusion on the activity of 5-LO. A23187 induces the translocation and attachment of 5-LO to membranes and thereby enhances the catalytic activity of the enzyme. Exogenous AA renders 5-LO independent of AA supply through phospholipases and FLAP. The triterpenic acids that exhibited potent inhibition of 5-LO in the cell-free assay also potently inhibited 5-LO product formation in this cellular model (Figure **54**). The effects at concentrations of 10 µM were even more evident in the cellular context.

Concentration-response analyses were carried out for the most potent compounds (Table **7**) and revealed IC_{50} values that were comparable or even below those of the most potent BAs, namely AKBA (IC_{50} = 3.2 µM) and KBA (IC_{50} = 2.8 µM). It was striking, that the most potent compound in this cellular assay (3α-OH-8,24-dien-TA) showed only moderate potency in the cell-free assay with an IC_{50} value that was about 20-fold increased.

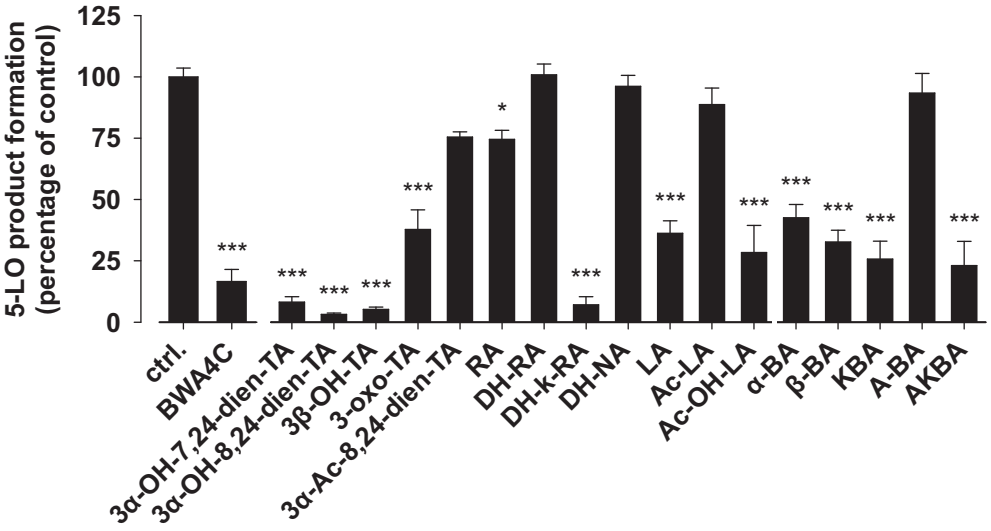


Figure 54: Effects of triterpenic acids on 5-lipoxygenase product formation in AA- plus A23187-stimulated human PMNL. PMNL (5×10^6 /ml in PGC buffer) were pre-incubated with vehicle (ctrl., DMSO), 0.3 µM BWA4C or the indicated triterpenic acids (10 µM) for 15 min at 37 °C. The cells were stimulated with AA 20 µM and A23187 2.5 µM for 10 min at 37 °C. The reaction was terminated and 5-LO product formation (5(S)-HETE, 5(S),12(S)-diHETE, LTB_4 and its all-trans isomers) was determined. Data are given as mean + S.E. of the percentage of the vehicle control with 889 ± 69.4 ng 5-LO products per 5×10^6 cells, n = 3 – 5, * $p < 0.05$, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey-Kramer *post hoc* test.

Table 7: IC_{50} values of triterpenic acids on 5-lipoxygenase product formation in AA- plus A23187-stimulated human PMNL. PMNL (5×10^6 /ml in PGC buffer) were pre-incubated with the indicated triterpenic acids for 15 min at 37 °C. The cells were stimulated with AA 20 µM and A23187 2.5 µM for 10 min at 37 °C. The reaction was terminated and 5-LO product formation (5(S)-HETE, 5(S),12(S)-diHETE, LTB_4 and its all-trans isomers) was determined. IC_{50} values were determined by fitting concentration response data to a four parameter logistic curve.

compound	IC_{50} [µM]	compound	IC_{50} [µM]
3α-OH-7,24-dien-TA	2.9	DH-k-RA	4.3
3α-OH-8,24-dien-TA	1.1	LA	4.0
3β-OH-TA	3.0	Ac-OH-LA	5.1
3-oxo-TA	7.1		
3α-Ac-8,24-dien-TA	26		

4.4.3 Effects of triterpenic acids from *Boswellia* species on 5-LO product formation in stimulated human whole blood

Human whole blood is commonly used to assess the inhibitory potential of 5-LO inhibitors in a physiologically relevant context. Human whole blood was treated with 3α-OH-8,24-dien-TA, 3β-OH-TA, 3-oxo-TA, 3α-Ac-8,24-dien-TA, DH-k-RA, LA and Ac-OH-LA at concentrations of 10 and 30 µM and stimulated with A23187 (30 µM). In contrast to their activity in isolated cells, no significant impact on 5-LO product formation was observed here (Figure **55**). Not only the effectiveness of the test compounds on 5-LO activity vanished in the whole blood model, but also the $cPLA_2\alpha$-inhibiting effects of Ac-OH-LA (see chapter 4.1).

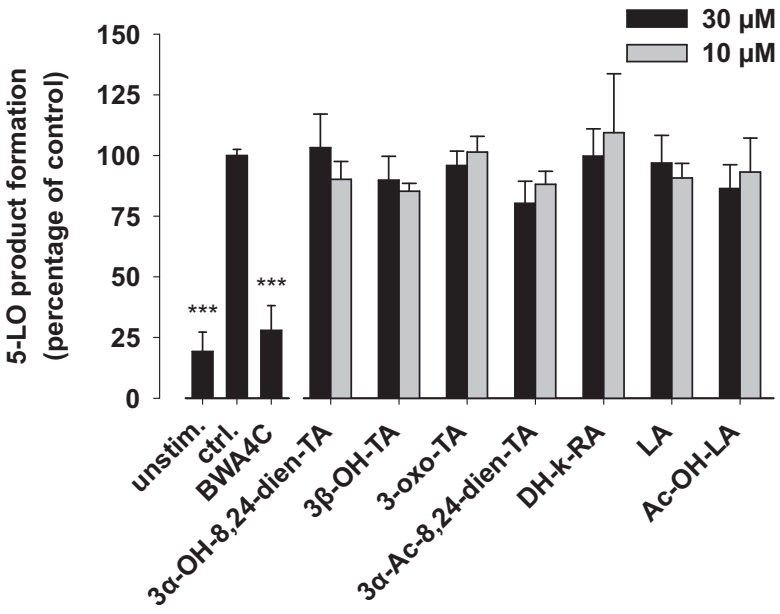


Figure 55: Effects of triterpenic acids on 5-lipoxygenase product formation in human whole blood. Heparinized human whole blood was pre-incubated with vehicle (ctrl., DMSO), 3 µM BWA4C and the indicated triterpenic acids (10 and 30 µM) for 10 min at 37 °C. The samples were stimulated with A23187 (30 µM) for 10 min at 37 °C, the incubation was stopped on ice and the content of 5-LO products (5(S)-HETE, 5(S),12(S)-diHETE, LTB_4 and its all-trans isomers) was determined by HPLC. Data are given as mean + S.E. of the percentage of the vehicle control with 106 ± 34.9 ng 5-LO products per ml blood, n = 3 – 6, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey-Kramer *post hoc* test.

4.5 Impact of triterpenic acids and frankincense extracts on neutrophil proteases CG and HLE

4.5.1 Effects of triterpenic acids and extracts from *Boswellia* species on Cathepsin G (CG) activity

CG was recently identified as a pharmacological target of BAs. As for 5-LO inhibition, the impact of other triterpenic acids on CG was only partially analyzed. For testing of the compounds, isolated human CG (1 µg/ml) was provided in HEPES buffered saline and the test compounds (10 and 1 µM) were added. The reaction was started by addition of the chromogenic substrate of CG (N-Suc-Ala-Ala-Pro-Phe-p-nitroanilide, 1 mM) and formation of p-nitroaniline (pNA) over time was monitored spectro-photometrically. The activity of the enzyme was determined to be 1.4 mU/µg in this setup. The synthetic CG inhibitor JNJ-10311795 almost totally suppressed pNA formation (Figure **56**). However, after incubation with 10 µM β-BA, the protease's activity was only repressed to 60% of the vehicle control, although β-BA was reported to inhibit CG with an IC_{50} value of 0.8 µM at the same conditions regarding enzyme amount and nature and concentration of the substrate [544]. Nevertheless, at 10 µM β-BA significantly inhibited CG and so did several other triterpenic acids, especially Ac-LA, which was markedly more potent than β-BA. 3-oxo-TA, 3α-Ac-7,24-dien-TA, DH-k-RA, DH-NA and LA inhibited CG to a similar extent as β-BA.

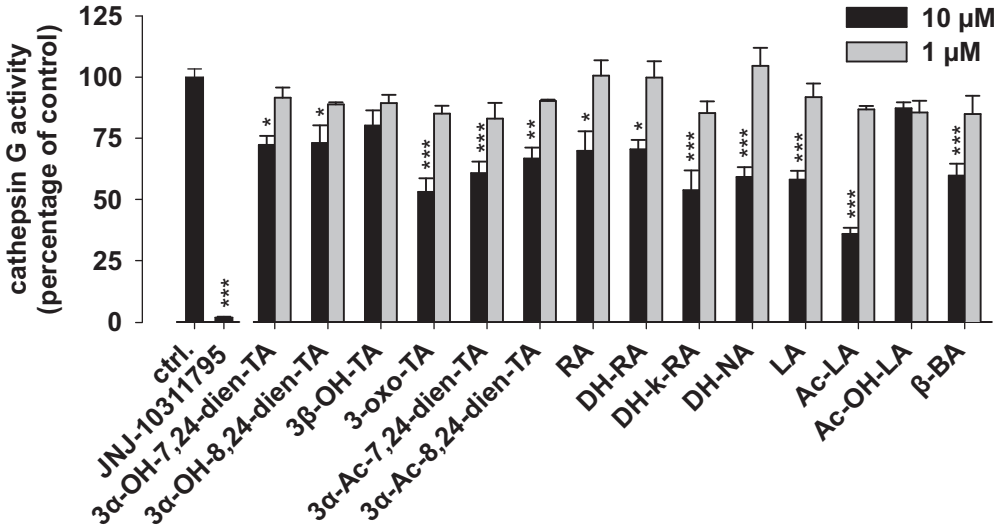


Figure 56: Impact of triterpenic acids on the activity of purified cathepsin G. 1 µg/ml of purified human CG in HEPES-buffered (100 mM) saline (500 mM) were incubated with vehicle (ctrl., DMSO), JNJ-10311795 (1 µM) or the indicated triterpenic acids (10 and 1 µM) for 10 min at 4 °C. The incubation was started by addition of 1 mM of the chromogenic substrate (N-Suc-Ala-Ala-Pro-Phe-p-nitroanilide) and the absorption of formed p-nitroaniline was recorded at 405 nm for 60 min (RT). Data are given as mean + S.E. of the percentage of the vehicle control with an activity of 1.4 ± 0.063 mU per µg CG. n = 3, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey HSD *post hoc* test.

CG is usually excreted by neutrophils upon stimulation e.g. with chemotactic stimuli. For this reason, PMNL were stimulated first with cytochalasin B, to facilitate the excretion and subsequently with the chemokine fMLP [574]. After stimulation, the cells were spun down and the supernatant was used as source of CG. This setup allows a more physiologic environment for CG. The substrate (N-Suc-Ala-Ala-Pro-Phe-p-nitroanilide) that was used in this assay is specifically cleaved by CG and therefore no cross-reactivity by other leukocyte-derived proteases is expected. The synthetic CG inhibitor JNJ-10311795 led to excessive suppression of CG activity here as well (Figure **57 A**). A-BA, which was described to possess an IC_{50} of 1.2 µM on the purified enzyme [544], was used as representative control of the BAs. But again, the BA was less potent, leading to 40% inhibition at a concentration of 10 µM. Especially the LAs and also 3-oxo-TA were more potent than A-BA, but only reduced CG activity to about 50 to 60% at 10 µM. As seen for the isolated enzyme, Ac-LA was the most potent triterpenic acid in this assay again. Although the enzymatic activity was lower in the protein mix excreted from PMNL, the inhibitory potential of the tested compounds was not markedly changed.

The acid fractions of extracts from different *Boswellia* species oleo-gum resins were tested in this assay as well (Figure **57 B**). The neutral fraction of *B. carteri* did not affect CG activity. In contrast, the acid fractions of all the tested extracts significantly inhibited the enzyme at 10 µg/ml. The most active extract was a. f. *B. papyrifera*, which was still significantly less potent than the

synthetic control inhibitor JNJ-10311795. At 10 µg/ml, a. f. *B. serrata* was markedly less potent than the respective extracts from the three other species.

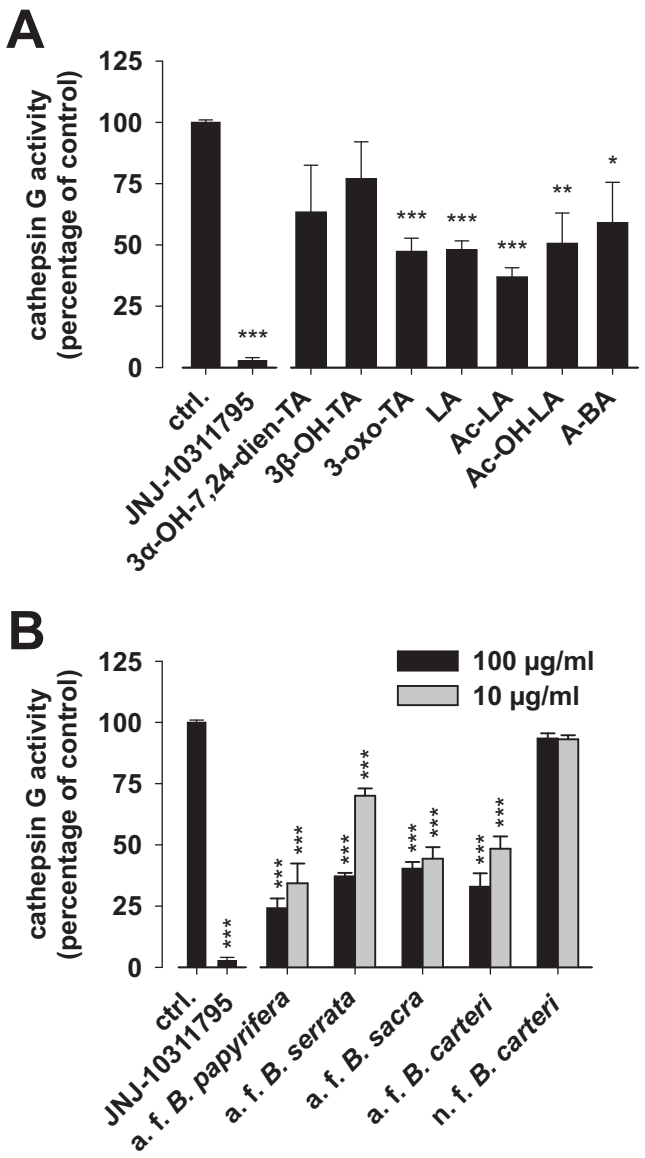


Figure 57: Interference of triterpenic acids and extracts from *Boswellia* species with cathepsin G in a protein mix directly excreted from neutrophils. Neutrophils were stimulated with 10 µM cytochalasin B (5 min, 37 °C) and 2.5 µM fMLP (5 min, 37 °C). After centrifugation, the protein content in the supernatant was determined and 7.5 µg protein per ml were diluted in HEPES-buffered (100 mM) saline (500 mM). This protein solution was incubated with vehicle (ctrl., DMSO), JNJ-10311795 (1 µM), the indicated triterpenic acids (10 µM, **A**) or the acid (a. f.) or neutral fraction (n. f.) of the extracts from the indicated *Boswellia* species oleo-gum resins (100 or 10 µg/ml, **B**) for 10 min at 4 °C. The incubation was started by addition of 1 mM of the CG-specific chromogenic substrate (N-Suc-Ala-Ala-Pro-Phe-p-nitroanilide) and the absorption of liberated p-nitroaniline was recorded at 405 nm for 60 min (RT). Data are given as mean + S.E. of the percentage of the vehicle control with an activity of 0.5 ± 0.056 mU per 7.5 µg proteins from PMNL excretion. n = 3 – 5, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey-Kramer *post hoc* test.

4.5.2 Impact of triterpenic acids and extracts from *Boswellia* species on human leukocyte elastase (HLE) activity

HLE is another protease that is secreted by leukocytes, e.g. upon stimulation with chemokines. AKBA and ursolic acid, another pentacyclic triterpenic acid, were reported to inhibit HLE activity with IC_{50} values of 15 and 2 µM, respectively [542]. The effect of several triterpenic acids and extracts from different *Boswellia* species on HLE activity was analyzed here. The same protein solution that was already used in the CG assay (see chapter 4.5.1) and that was obtained by stimulation of PMNL with cytochalasin B and fMLP, was used as source for HLE. For analysis of HLE activity, the specific substrate N-MeOSuc-Ala-Ala-Pro-Val-p-nitroanilide was used to avoid cross-reactivity of other leukocyte-derived proteases. The synthetic HLE inhibitor sivelestat (50 nM) potently inhibited the protease's activity (Figure **58 A**). In contrast, AKBA (10 µM) was rather ineffective and also ursolic acid (10 µM) only suppressed HLE activity to 57%, thus markedly less potent than reported. Most of the triterpenic acids that were tested showed more efficient inhibition of HLE activity than AKBA. The impact of 3α-Ac-8,24-dien-TA, RA and Ac-LA was similar to that of ursolic acid. Nevertheless, it was significantly differing from that of the control inhibitor sivelestat. In addition, several extracts form *Boswellia* species oleo-gum resins were tested in this assay (Figure **58 B**). At high concentrations (100 µg/ml), the acid fraction of the extracts from *B. papyrifera* and *B. serrata* almost reached the inhibitory effect of sivelestat. However, at lower concentrations (10 µg/ml) the effect was clearly attenuated, though still significant. Interestingly, the neutral fraction of the extract from *B. carteri* caused significant inhibition of HLE at 100 µg/ml as well, which was comparable to the acid fraction of the same extract.

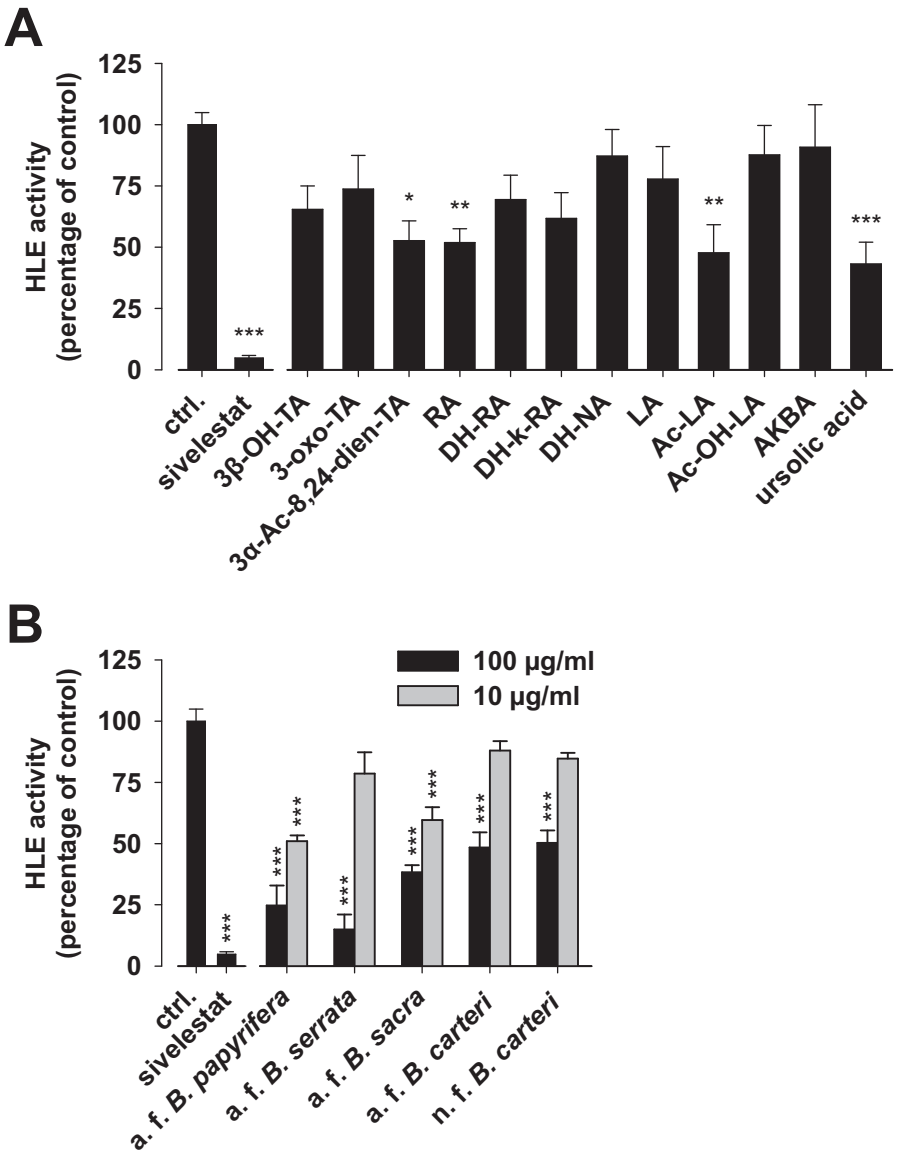


Figure 58: Interference of triterpenic acids and extracts from *Boswellia* species with human leukocyte elastase (HLE) in a protein mix directly excreted from neutrophils. Neutrophils were stimulated with 10 µM cytochalasin B (5 min, 37 °C) and 2.5 µM fMLP (5 min, 37 °C). After centrifugation, the protein content in the supernatant was determined and 7.5 µg protein per ml were diluted in HEPES-buffered (100 mM) saline (500 mM). This protein solution was incubated with vehicle (ctrl., DMSO), sivelestat (50 nM), the indicated triterpenic acids (10 µM, **A**) or the acid (a. f.) or neutral fraction (n. f.) of the extracts from the indicated *Boswellia* species oleo-gum resins (100 or 10 µg/ml, **B**) for 10 min at 4 °C. The reaction was started by addition of 0.1 mM of the HLE-specific chromogenic substrate (N-MeOSuc-Ala-Ala-Pro-Val-p-nitroanilide) and the absorption of liberated p-nitroaniline was recorded at 405 nm for 10 min (RT). Data are given as mean + S.E. of the percentage of the vehicle control with an activity of 1.5 ± 0.41 mU per 7.5 µg proteins from PMNL excretion. n = 4 – 6, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey-Kramer *post hoc* test.

4.6 Effects of triterpenic acids from *Boswellia* species on glucocorticoid receptor signalling

Boswellia preparations are used in traditional and modern medicine to treat diseases with an inflammatory background – such as autoimmune diseases or chronic inflammation. The application widely coincides with that of glucocorticoids and structural similarities between glucocorticoids and certain triterpenic acids are obvious. This raised the question, whether compounds in extracts from *Boswellia* species could directly act as ligands of the glucocorticoid receptor. For this reason, a glucocorticoid receptor response element (GRE) luciferase reporter assay was performed. A549 cells were chosen to be transiently transfected with the reporter or control constructs, since the glucocorticoid receptor is highly involved in transcriptional signalling in this cell line [591]. Three approaches were performed to control proper transfection and readout of the luciferase activity. The first approach (positive control) contained constitutively expressing firefly and *Renilla* luciferase constructs and a constitutively expressing GFP construct. The second approach (reporter approach) contained the inducible glucocorticoid receptor responsive firefly luciferase reporter construct and a constitutively expressing *Renilla* luciferase construct. The third approach (negative control) resembled the second one, but contained a non-inducible firefly luciferase reporter lacking the transcriptional response element (GRE). The first approach was used to assure the successful transfection of the cells, which was checked by fluorescence microscopy for GFP in the intact cells and by assessing the activity of constitutively expressed firefly and *Renilla* luciferases after lysis of the cells (ratio of firefly to *Renilla* luciferase activity 0.713 ± 0.0496). Actually, only part of the cells that were observed under the light microscope (Figure **59 A**) could also be visualized by fluorescence microscopy (Figure **59 B**) and thus were successfully transfected.

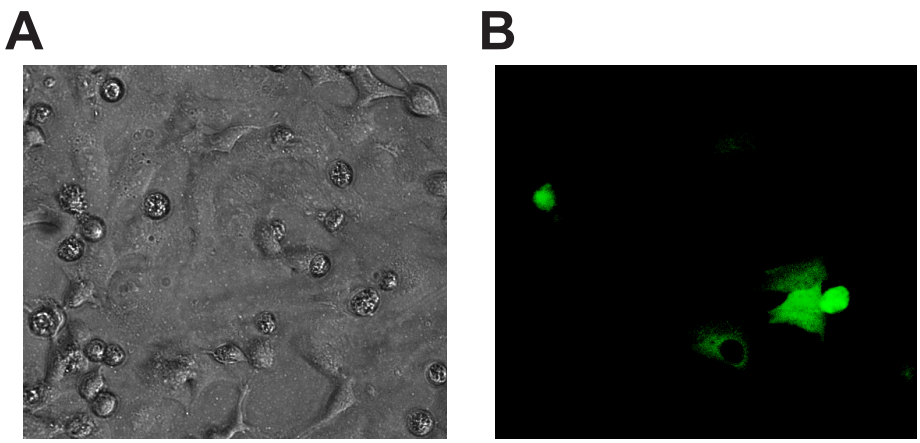


Figure 59: Expression of GFP in A549 cells after transfection with luciferase and GFP constructs. A549 cells (2×10^4 cells in 100 µl RPMI 1640 medium supplemented with 2% FCS) were transfected with constructs (100 ng) constitutively expressing GFP and firefly and *Renilla* luciferases. After 24 h (37 °C, 6% CO_2), medium was changed to RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. After 16 h, the same sector of cells was examined under a light microscope (**A**) or by fluorescence microscopy (**B**) excitating at 470 nm and recording emission at 515 nm.

After transfection and recovery of the cells, the test compounds were added and cells were further incubated for 6 h. For each reporter approach, one negative control was used containing the respective test compound to identify background reporter activity and unspecific effects that were not related to glucocorticoid receptor signalling. The incubation was stopped and the luciferases were released from the cells by passive cell lysis. The luciferases' activity was determined using a dual-luciferase reporter assay system (Promega GmbH, Mannheim, Germany). Constitutively expressed *Renilla* luciferase served as internal control to normalize fluctuations in transfection efficiency and effects of the test compounds on cell viability. As expected, dexamethasone significantly stimulated the relative luciferase activity about 7.5-fold (ratio of the reporter luciferase activity to the constitutive luciferase activity related to the same ratio of the negative control) (Figure **60**). On the contrary, none of the triterpenic acids significantly induced luciferase activity at a concentration of 30 µM. The neutral triterpene amyrin (30 µM) or the neutral fraction of the extract from *B. carteri* (30 µg/ml) were ineffective in modulating the activity of the glucocorticoid receptor on the transcriptional response element as well.

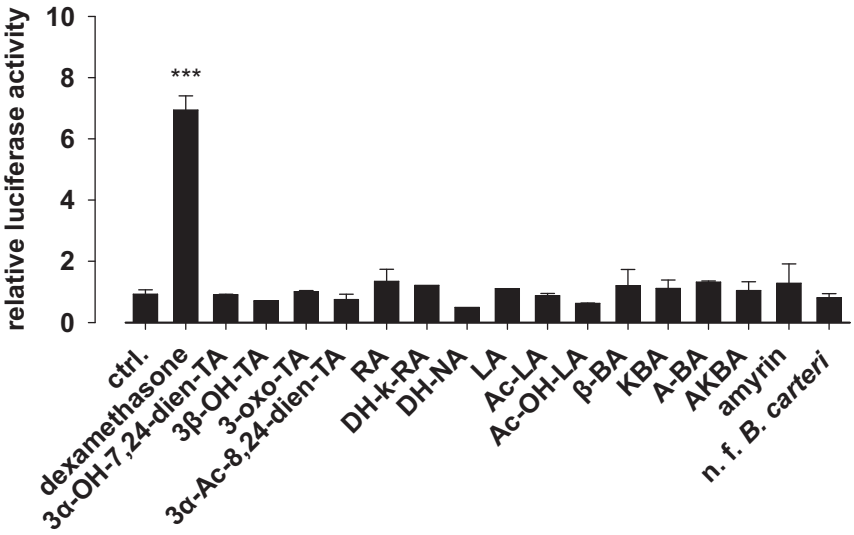


Figure 60: Relative glucocorticoid receptor response element-dependent luciferase activity in transfected A549 cells after treatment with diverse triterpenes and the neutral fraction of the extract from *B. carteri* oleo-gum resin. A549 cells (2×10^4 cells in 100 µl RPMI 1640 medium supplemented with 2% FCS) were transfected either with 100 ng of a mixture of an inducible glucocorticoid receptor responsive firefly luciferase reporter construct and a constitutively expressing *Renilla* luciferase construct (reporter approach) or with a mixture of a non-inducible firefly luciferase reporter construct and a constitutively expressing *Renilla* luciferase construct (negative control). After 24 h (37 °C, 6% CO_2), medium was changed to RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. After 16 h the cells were treated with vehicle (ctrl., DMSO), 100 nM dexamethasone, the indicated triterpenes (30 µM) or the neutral fraction of the extract from *B. carteri* oleo-gum resin (n. f. *B. carteri*, 30 µg/ml). 6 h later, the reaction was stopped by rinsing the cells with PBS buffer and passive cell lysis. The luciferases' activity was monitored using a dual-luciferase reporter assay system. Data are given as mean + S.E. of the relative luciferase activity (ratio of firefly luciferase activity to *Renilla* luciferase activity in the reporter approach related to the same ratio in the negative approach). n = 2 – 4, *** $p < 0.001$ vs. ctrl., one-way ANOVA followed by Tukey-Kramer *post hoc* test.

5 Discussion

5.1 Effect of lupeolic acids on cPLA$_2$α activity and evaluation of the applied test systems

Inhibition of cPLA$_2$α has been proposed as pharmacological strategy in the treatment of diverse inflammatory diseases like asthma [592], inflammatory skin diseases [244, 593-594] or arthritis [108, 595]. In fact, cPLA$_2$α activity is upregulated during inflammation and inhibition of this enzyme leads to effective reduction of pro-inflammatory PGs, LTs and PAF, without affecting lipid homoeostasis that is predominantly driven by alternative phospholipases [596]. Although preclinical data with cPLA$_2$α inhibitors were promising, the potential of these compounds as therapeuticals in inflammatory diseases could not be confirmed in clinical trials yet. This is at least partly due to the fact that many cPLA$_2$α inhibitors are not drug-like substances. For instance, the first generation of inhibitors showed high cytotoxicity because of their amphiphilic structure [230]. The recently developed synthetic cPLA$_2$α inhibitors are less cytotoxic but show only poor bioavailability [240].

In this thesis, a rich variety of structurally distinct triterpenic acids from *Boswellia* species were tested in a cell-free assay on cPLA$_2$α activity, including LAs, which have been discovered in frankincense only in the last decade [500-502] and whose pharmacological effects are widely unexplored. Assays using vesicular membranes as substrate are commonly utilized to illustrate direct inhibition of the cPLA$_2$α-membrane interaction and the potency of established cPLA$_2$α inhibitors in these test systems closely correlates with that in natural membrane models [597]. The screening of triterpenic acids highlighted the impact of Ac-OH-LA on cPLA$_2$α. Other triterpenic acids, also LA analogues lacking the C-28-hydroxy moiety, were only partially effective. Obviously, the C-28-hydroxy-moiety essentially increases the interference with cPLA$_2$α. Apart from being a lipophilic acid, Ac-OH-LA is structurally distinct from other known cPLA$_2$α inhibitors. In particular with regard to its molecular weight, Ac-OH-LA is a rather small molecule. It should be noted that inhibition of isolated cPLA$_2$α in a cell-free model does not prove direct inhibition of the catalytic activity of cPLA$_2$α. Inhibition could also result from impaired interaction of the enzyme with the phospholipid interface, an effect that has been observed for Choline-type inhibitors for example [231]. As cPLA$_2$α is an interface-activated enzyme (see chapter 2.2.2.4), inhibition might also result from allosteric binding to cPLA$_2$α, which impairs interfacial activation of the enzyme.

Inhibition of cPLA$_2$α by Ac-OH-LA was verified in platelets, monocytes and PMNL as cellular test systems. Upon stimulation with A23187, all these cells release AA in a cPLA$_2$α-dependent manner [65, 228, 598], which was confirmed in the present work by the complete reversal of A23187-stimulated AA release by the cPLA$_2$α control inhibitor. Similarly to the cell-free assays, also in the cellular models the inhibitory potential of Ac-OH-LA was higher in comparison to the LA analogues without C-28-hydroxy function. In the different cell types, a basal AA release was also observed in the absence of ionophore stimulation. This basal AA release was less pronounced in PMNL than in platelets or monocytes, probably as a result of cell-specific membrane-remodelling processes. For example, sPLA$_2$ (group II) activity [580] or indirect decomposition of membranous phospholipids through phospholipase C and diglyceride lipase [581] were identified as alternative mechanisms leading to AA release in platelets. Also in monocytes, AA can alternatively be supplied through sPLA$_2$ [598]. However, the basal AA level was neither affected by Ac-OH-LA nor by the cPLA$_2$α control inhibitor, which implies selectivity of Ac-OH-LA for AA release mediated by cPLA$_2$α after stimulation and suggests no general interference with membrane homoeostasis.

The inhibition of AA release by Ac-OH-LA resulted in the subsequent reduction of AA metabolites produced in A23187-stimulated platelets, PMNL and monocytes. This is reflected by similar IC$_{50}$ values on the level of AA release and metabolite formation. Interestingly, unlike the other investigated metabolites, 12-HHT formation in platelets was only partially inhibited by the cPLA$_2$α control inhibitor. We suggest that A23187-stimulated 12-HHT production by COX-1 does not exclusively depend on AA provided by cPLA$_2$α, but also on alternative sources. On the contrary, Ac-OH-LA completely suppressed 12-HHT formation in a similar way as ibuprofen, which indicates an additional effect on COX-1 activity, as confirmed in a cell-free COX-1 assay (see chapter 4.3.4).

The inhibition of AA metabolite production (e.g. 12-HHT and 12-HETE in platelets and 12-HETE in monocytes and PMNL) by Ac-OH-LA was significantly reversed by the addition of exogenous AA, which confirms inhibition of AA supply through cPLA$_2$α. Interestingly, we found that 5-LO product synthesis in monocytes and PMNL was instead not fully restored by exogenous AA, which is apparently related to direct interference of Ac-OH-LA and LA with 5-LO (see chapter 4.4). Similarly, 12-HHT formation in Ac-OH-LA-treated platelets and monocytes was only partially restored by exogenous AA, which might be due to direct interference with COX-1 (see above and chapter 4.3.4). On the other hand, we observed that also the cPLA$_2$α control inhibitor partially suppressed AA-stimulated 12-HHT formation. This might be due to unspecific interference of the cPLA$_2$α inhibitor with COX-1 activity. Alternatively, it was shown that

reduction of prostanoid synthesis in monocytes could result from coordinated functioning of COX and cPLA$_2$α [599]. Thus, uncoupling of cPLA$_2$α and COX may not be completed by supplementation of exogenous AA (20 µM) and the inhibition of cPLA$_2$α by Ac-OH-LA or the control inhibitor may still influence 12-HHT production here.

Similarly, 5-LO product formation in PMNL after incubation with the specific cPLA$_2$α control inhibitor and stimulation with A23187 plus AA (20 µM) was still inhibited by about 50%. This finding is in agreement with previous results with the related cPLA$_2$α inhibitor pyrrophenone [600] and once again might be caused by the two phenomena: (i) the control inhibitor might directly inhibit 5-LO activity when applied in micromolar concentrations, as shown for pyrrophenone [600]; (ii) cPLA$_2$α and 5-LO are not completely uncoupled by exogenous AA. We observed that the cPLA$_2$α inhibitor (5 µM) suppressed 5-LO product formation by almost 50% in a cell-free assay, which indicates that the first hypothesis (direct inhibition of 5-LO) is correct. However, when monocytes were stimulated with A23187 plus AA, inhibition of 5-LO product formation by the cPLA$_2$α inhibitor was not significant, which on the contrary suggests minor direct 5-LO inhibition in the cellular context. In analogy to the cPLA$_2$α-COX coupling in platelets (see above), the differences observed in PMNL and monocytes might be based on differential coupling of phospholipases and 5-LO in these cell types. In fact, previous studies support the coupling of 5-LO to alternative sources of AA in monocytes [599], whereas 5-LO product formation in PMNL is prone to inhibition of cPLA$_2$α [600]. Alternatively, the cPLA$_2$α control inhibitor might unspecifically interfere with structures that regulate 5-LO product formation in a cell-specific manner.

Aggregation measurements in platelets were carried out to assess the functional relevance of cPLA$_2$α inhibition by Ac-OH-LA. Collagen stimulation in platelets involves cPLA$_2$α-, COX-1- and TX synthase-dependent production of TXA$_2$, which is required for the induction of receptor-mediated aggregation [577] (Figure **61**). In addition to collagen, we also used the TXA$_2$ analogue U46619 as alternative stimulus to discriminate effects during TXA$_2$ synthesis and effects downstream of TXA$_2$ formation. As expected, the COX inhibitor indomethacin and the cPLA$_2$α control inhibitor did not affect U46619-induced platelet aggregation. On the other hand, both inhibitors substantially diminished aggregation of collagen-stimulated cells. Interestingly, aggregation was totally blocked by inhibition of COX, whereas inhibition of cPLA$_2$α still allowed minor aggregation. As stated above, this might be due to alternative, cPLA$_2$α -independent supply of AA leading to COX-1 product (and thus TXA$_2$) formation. After treatment of platelets with 10 µM Ac-OH-LA (a concentration that led to extensive inhibition of AA release), collagen- but not U46619-induced aggregation was significantly inhibited, supporting the hypothesis that

Ac-OH-LA acts via inhibition of cPLA$_2$α. Further increase of the Ac-OH-LA concentration decreased aggregation beyond the level of the cPLA$_2$α control inhibitor, which was also accompanied by inhibition of U46619-induced aggregation. Apparently, elevated Ac-OH-LA concentrations suppress platelet aggregation by action on both cPLA$_2$α and an additional target downstream of TXA$_2$ synthesis. The IC$_{50}$ value for inhibition of collagen-induced aggregation was higher than that found for inhibition of A23187-induced AA release. Though not immediately understood, this difference may be related to altered kinetic properties of cPLA$_2$α resulting from phosphorylation events. In fact, in addition to elevation of intracellular Ca^{2+} levels, collagen increases cPLA$_2$α activity by phosphorylation at Ser^{505} and Ser^{727} through p38 MAPK and MNK1/PRAK1, respectively [178, 578, 601]. Surprisingly, at concentrations of 10 µM or above LA and Ac-LA (lacking the C-28-hydroxy group) totally inhibited both collagen- and U46619-induced aggregation. A similar behaviour was already found for 11-keto-BAs, which was attributed to inhibition of Ca^{2+} mobilization [602].

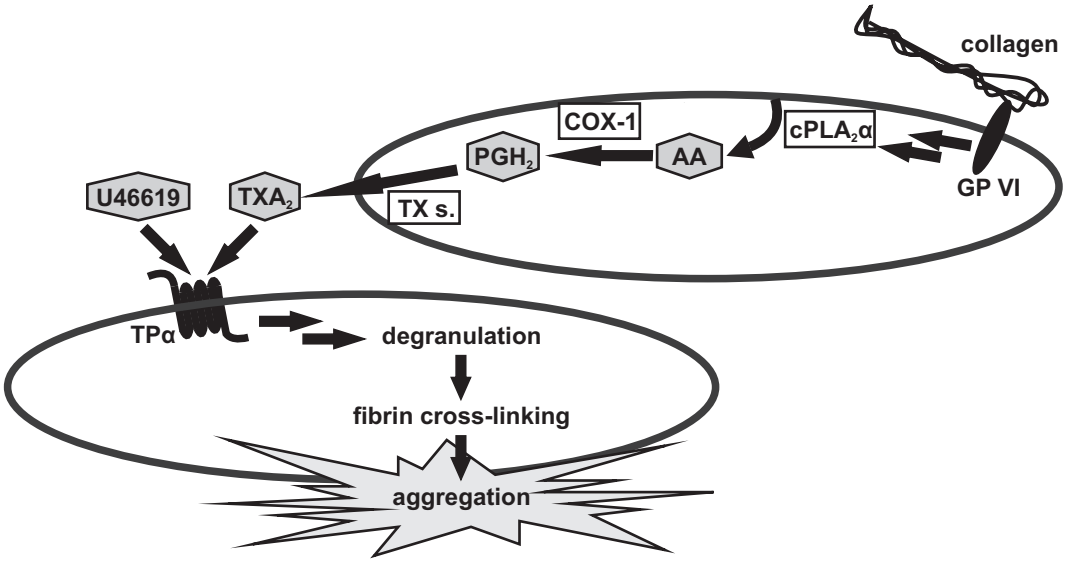


Figure 61: Collagen- and U46619-induced platelet aggregation. Thromboxane synthase (TX s.).

Ac-OH-LA did not significantly inhibit AA metabolite production in A23187-stimulated human whole blood. Similarly, BAs failed to inhibit 5-LO in human whole blood, which was related to extensive protein binding of BAs [603]. High binding to plasma proteins might be reasonable for Ac-OH-LA as well. In fact, we observed a reduction of the inhibitory potential of Ac-OH-LA on isolated cPLA$_2$α in the presence of BSA (starting at 1 mg/ml).

In summary, Ac-OH-LA is a potent inhibitor of cPLA$_2$α activity, leading to reduced AA release and AA metabolite production in different blood cells. Functionally, this interaction results in reduction of platelet aggregation. The effects on AA metabolite production were impaired in whole blood, probably due to plasma protein binding. *In vivo* studies so far were only performed

with preparations from *Boswellia* species, but their total content of Ac-OH-LA, though not yet quantified, is presumably rather low. Thus, the contribution of Ac-OH-LA to the anti-inflammatory effects of frankincense extracts is probably limited. Nevertheless, the identification of Ac-OH-LA as cPLA$_2$α inhibitor represents a significant advance in the field because it is both a natural compound and a relatively small molecule. It might serve as lead structure for the development of new synthetic cPLA$_2$α inhibitors with improved pharmacokinetic properties. To this aim, investigation in a more physiological context (pharmacokinetic studies, *in vivo* models of inflammation) would be valuable.

5.2 Effects of tirucallic acids on arachidonic acid mobilization in human platelets

We show here that TAs cause massive release of AA from intact platelets by kinase- and Ca^{2+}-dependent activation of cPLA$_2$α. TAs are tetracyclic triterpenes that make up about 14% and 23% of the acid fraction extracted from *B. papyrifera* and *B. serrata* resin, respectively, with 3-oxo-TA as major ingredient accounting for 7.5 to 10% [498]. Despite of their high abundance, only few reports addressed TAs. For example pro-apoptotic effects in cancer cells [557] and the modulation of 5-LO product formation in PMNL [538] by TAs were reported. TAs were shown to stimulate A23187-induced 5-LO product formation in intact PMNL when added right before challenge with A23187, which was attributed to direct activation of 5-LO via the MEK/ERK pathway. On the other hand, stimulatory effects on 5-LO product formation through ERK/p38 MAPK activation and Ca^{2+} mobilization were also found in BA-treated PMNL (AKBA), which was ascribed to enhanced AA supply instead of direct stimulation of 5-LO [536, 579]. Similar mechanisms may be assumed for TAs in PMNL as well. In platelets, BAs were shown to trigger Ca^{2+} mobilization and ERK, p38 MAPK and Akt phosphorylation [541]. β-BA caused cPLA$_2$α-mediated AA release independently of Ca^{2+} and without direct interaction with cPLA$_2$α. This AA release was suppressed by Src family kinase and PI3 kinase inhibitors but not by inhibitors of ERK or p38 signalling, and only in the presence of Ca^{2+} [539].

Similarly, we observed that the TA-induced AA release and subsequent metabolization in platelets occurred independently of Ca^{2+}. Interestingly, relative AA release by TAs was even enhanced in the absence of Ca^{2+}. Though the four analyzed TAs only slightly differ in their structure, this effect was particularly evident for Ac-TA. The differential efficacy of the respective TAs upon Ca^{2+} deprivation indicates the superposition of different mechanisms leading to AA release. On the AA metabolite level, the stimulatory effects were even more pronounced. Presumably, the two-hour labelling with [^{3}H]-labelled AA in the AA release assay renders the cells in part refractory to

stimuli such as TAs or A23187. Upon incubation with TAs, the concentration-dependent metabolite production developed very fast with maximal stimulation of 12-HHT production after 30 to 60 sec and time-dependent increase of 12-HETE formation over 1 h. As 12-HHT is a product of COX-1 and this enzyme undergoes auto-inactivation within a few minutes after substantial AA supply, the cessation of 12-HHT formation is a COX-specific observation [10]. The fact that auto-inactivation emerged within the same time span irrespective of the intensity of the stimulus appears however unexpected. On the other hand, usually upon cell stimulation, only small subsets of COX enzymes are activated [248]. Thus, stimulation by each TA recruits a definite population of COX enzymes that are fully active in each case and thus undergo inactivation within similar time scales. Unlike COX or 5-LO, 12-LO does not show auto-inactivation [426]. Consequently, 12-HETE formation is an appropriate indicator for sustained AA release generated by TAs. As TA-induced 12-HHT and 12-HETE formation were blocked after pre-treatment with the $cPLA_2\alpha$ inhibitor RSC-3388, AA mobilization most likely is carried out by this phospholipase. On the other hand, $cPLA_2\alpha$-triggered stimulation of AA release through other PLA_2s could also account for the efficacy of RSC-3388 [70]. But since fast and extensive mobilization of AA through $iPLA_2$ seems unlikely and $sPLA_2$ is dependent on relatively high levels of Ca^{2+}, their contribution to the fast and Ca^{2+}-independent AA release elicited by TAs appears implausible.

Surprisingly, 12-HHT formation in platelets was generally stimulated after treatment with the $cPLA_2\alpha$ inhibitor. As already mentioned in the previous chapter, 12-HHT formation in platelets is only partially dependent on $cPLA_2\alpha$. After blocking of $cPLA_2\alpha$, alternative sources of AA predominate that seem to couple with COX-1 rather than with 12-LO, which might be due to the differential localization of COX and 12-LO at low intracellular Ca^{2+} levels.

Investigations analyzing the subcellular distribution of $cPLA_2\alpha$ confirmed the stimulatory effects of TAs on $cPLA_2\alpha$ activity. Considerable translocation of $cPLA_2\alpha$ to the membranous fraction indicates enhanced interaction of the enzyme with the membrane surface that is normally mediated by increased intracellular Ca^{2+} levels [128] or by phosphorylation of $cPLA_2\alpha$ [140]. Membrane translocation correlated with the stimulatory effects on 12-HETE formation.

The acylphloroglucinol hyperforin from *Hypericum perforatum* elicits similar effects in platelets as TAs: It induces $cPLA_2\alpha$-mediated, but Ca^{2+}-independent AA release and $cPLA_2\alpha$ translocation [569]. These effects were ascribed to the induction of $cPLA_2\alpha$ phosphorylation and the insertion of hyperforin into membranes, facilitating the access of $cPLA_2\alpha$ to its substrate. In fact, the structural resemblance of the TAs and cholesterol would support potential interference by membrane insertion. On the other hand, TAs carry a hydrophilic carboxylic group centrally located on their lipophilic scaffold, and therefore intercalation in the membrane might be thermodynamically

unfavoured. Anyway, the effect of TAs on cPLA$_2$α activity was investigated using membranes featuring different rigidity and dissimilar phospholipid headgroups. Although cPLA$_2$α activity varied considerably in these membrane models, TAs did not induce AA release in any system, and thus a noteworthy direct effect on polarity, accessibility or crystallinity of membranes seems improbable. Nevertheless, TAs may also interact with ceramides, diacylglycerol, C1P, PIPs or metabolizing enzymes thereof (see chapter 2.2.2.4).

A crucial initiator of cPLA$_2$α activity is the mobilization of intracellular Ca^{2+} (Figure **62**), which was already shown to be effected by BAs in platelets [541] and might contribute to TA-induced cPLA$_2$α activity – at least in the presence of Ca^{2+}. In fact, TAs provoked a fast and sustained boost of intracellular Ca^{2+} levels, which approximately correlated with 12-HHT and 12-HETE formation in the presence of Ca^{2+}. Compared to stimuli like A23187, thapsigargin or thrombin, these Ca^{2+} fluxes were modest, but definitely reached levels that are necessary for the induction of cPLA$_2$α membrane translocation and cPLA$_2$α activity, especially since the increase in the Ca^{2+} levels was sustained [156].

Phosphorylation events are essential for cPLA$_2$α activity in platelets upon stimulation with physiological stimuli such as collagen or thrombin [172] (Figure **62**). As CaMKII- and MNK1/MSK1/PRAK1-induced phosphorylation of Ser^{515} and Ser^{727} are frequently linked to MAPK-induced phosphorylation of Ser^{505} [178, 182, 186-187], Western blot analyses concentrated on the activation of JNK, ERK and p38 MAP kinases. In Ca^{2+}-containing buffer, stimulation with TAs led to phosphorylation of p38 and to a minor extent of JNK, indicating potential activation of cPLA$_2$α via phosphorylation at Ser^{505}. Nevertheless, the impact on p38 phosphorylation after treatment with the different TAs did not exactly correlate with the increase in 12-HETE formation. Although the assessment of TA-induced p38 phosphorylation was impeded in EDTA- and BAPTA-AM-treated cells, at least 3α-OH-TA and Ac-TA visibly increased p38 phosphorylation, proposing effectiveness of this route of cPLA$_2$α activation in a Ca^{2+}-depleted environment as well. Similar to 12-HETE formation and cPLA$_2$α membrane translocation, the effect of 3α-OH-TA on p38 phosphorylation was increased in the absence of Ca^{2+}.

Direct analysis of the phosphorylation of cPLA$_2$α at Ser^{505} confirmed the phosphorylation after treatment with 3-oxo-TA and Ac-TA in a Ca^{2+}-containing medium and after treatment with all TAs in the absence of Ca^{2+}, which is in line with the activation of p38 and JNK MAPK.

In a kinase inhibitor approach, we confirmed the involvement of p38 MAPK and additionally found CaMKII to mediate TA-induced AA metabolite production. The effect of the p38 MAPK inhibitor on TA-induced 12-HETE formation was even more pronounced after chelation of Ca^{2+}.

Notably, the importance of MAPK-induced cPLA$_2$α phosphorylation stringently depends on the intracellular Ca^{2+} levels. For instance, inhibitors of p38 or ERK do not affect cPLA$_2$α activity in thrombin-stimulated platelets due to sustained Ca^{2+} mobilization [175, 601, 604], but phosphorylation of Ser^{505} by MAPK clearly controls cPLA$_2$α activity at low Ca^{2+} levels [140, 601]. CaMKII stimulates cPLA$_2$α activity, which is accompanied by phosphorylation of Ser^{515} [173] but also occurs independently of Ser^{515} [185]. As CaMKII activity is closely connected to Ca^{2+} signalling [605], its effect on cPLA$_2$α activity after treatment with TAs might simply result from Ca^{2+} mobilization. On the other hand, the CaMKII inhibitors maintained their suppressive effect in Ca^{2+}-deprived cells, suggesting Ca^{2+}-independent effects to trigger CaMKII-driven activation of cPLA$_2$α. In smooth muscle cells, CaMKII was shown to be linked to MAPK activation and thereby led to phosphorylation of cPLA$_2$α at Ser^{505} [187]. However, TA-induced p38 phosphorylation was not blocked by the CaMKII inhibitor KN-93 in platelets (data not shown), which excludes a direct linkage of these kinases here.

In summary, TAs Ca^{2+}-independently induced the activation of cPLA$_2$α in platelets, whereas no effect was seen in PMNL. This might be based on the fact that the threshold for activation of cPLA$_2$α in PMNL is higher than in platelets. The four TAs tested affected Ca^{2+} mobilization and kinase cascades to different extents, both of which are known to induce cPLA$_2$α activation. The stimulatory effect of TAs in platelets could be of physiologic relevance since TAs represent major compounds in extracts from *B. papyrifera* and *B. serrata*. Frankincense formulations are usually applied in chronic inflammatory diseases but the platelet-activating properties of TAs could abet unfavourable effects. For example, enhanced platelet activation is associated with inflammatory diseases such as atherosclerosis [606], rheumatoid arthritis [607], inflammatory bowel diseases [608] and psoriasis [609-610]. On the other hand, also BAs elicit stimulating effects on AA metabolism in platelets [539] and PMNL [579], but the inhibitory effects appear to dominate in more physiologic systems and *in vivo*. The same might apply to TAs as they also represent potent inhibitors of AA metabolizing enzymes (see chapters 4.3 and 4.4). For instance, *in vivo* application of 3-oxo-TA, the most abundant TA in *B. papyrifera* and *B. serrata* extracts, indicated preponderance of inhibitory effects on eicosanoid synthesis (see chapter 4.3.6). Also in stimulated monocytes or human whole blood, inhibitory effects of 3-oxo-TA mostly prevailed. Anyway, for conclusive evaluation of the relevance of stimulatory effects *in vivo*, the plasma levels of TAs after the application of frankincense extracts must be assessed.

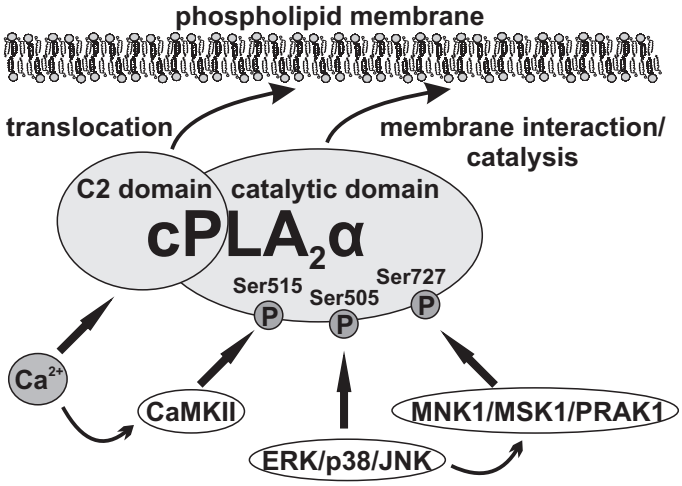


Figure 62: Regulation of cPLA$_2$α.

5.3 Interaction of triterpenic acids and frankincense extracts with PGE_2 biosynthesis

PGE_2 is a central mediator in inflammation, governing oedema formation, pain sensitization and fever [8]. Anti-inflammatory actions of NSAIDs are essentially attributed to the suppression of inflammatory PGE_2. Anyway, these commonly used drugs evoke severe side effects, especially when they are applied in the long-term therapy of chronic inflammatory diseases such as rheumatoid arthritis. Gastrointestinal toxicity is mediated by inhibition of COX-1-derived PGE_2 in the gastric mucosa and was overcome by the development of selective COX-2 inhibitors. Unfortunately, selective inhibition of COX-2 favours the synthesis of COX-1-derived pro-aggregatory TXA_2, whereas PGI_2 as anti-aggregatory and vasodilatory mediator is suppressed, leading to increased risk of cardiovascular complications [16]. As mPGES-1 is upregulated upon stimulation with pro-inflammatory cytokines and thereby essentially gives rise to PGE_2 synthesis in inflammation, its inhibition promises efficient suppression of PGE_2 synthesis in an inflammatory context, avoiding effects on other prostanoids or physiologic PGE_2 synthesis [312]. Several natural compounds were identified as inhibitors of mPGES-1, such as curcumin [611], garcinol [612], myrtucommulone [613], epigallocatechin-3-gallate [614] and BAs [511]. Here, several extracts from different *Boswellia* species were assayed on their inhibitory potential on mPGES-1 activity in a cell-free approach. Since numerous established mPGES-1 inhibitors are lipophilic acids, special attention was paid to the acid fractions from frankincense extracts. In fact, the neutral fraction from *B. carteri* was ineffective, whereas all acid fractions demonstrated concentration-dependent inhibition of mPGES-1. Bio-guided fractionation of the most potent acid fraction (a. f.) from *B. papyrifera* revealed 3-oxo-TA as the most effective component. Other

triterpenic acids also showed inhibition of mPGES-1 activity with similar or even higher potency. However, in the extract from *B. papyrifera*, 3-oxo-TA was the most abundant of these potent inhibitors and thereby is most likely responsible for its superior efficiency. Nevertheless, the concentration-response-curve of 3-oxo-TA coincided with the one of the whole extract, which suggests the contribution of even more potent compounds to the effectiveness of the extract. While BAs were reported to have IC_{50} values of 3 µM or higher [511], several triterpenic acids that were analyzed in this study were more effective, with the two Ac-TA derivatives being the most potent compounds (IC_{50} = 0.4 µM). The potency of these Ac-TAs was unaffected by the conformational alteration of the tetracyclic ring system that results from relocation of the double bond from position 7 to 8. Regarding the 3α-hydroxy derivatives, this conformational shift led to different concentration-response curves, with the IC_{50} value of the flexed 7,24-diene derivative being higher than that of the 8,24-diene. Notably, the latter suppressed PGE_2 formation with higher potency than the reference drug MK-886. Similar inhibition, though at high concentrations, was only accomplished by the a. f. from *B. papyrifera* extract. Residual PGE_2 formation that is not suppressed by selective mPGES-1 inhibitors could arise from enzymatic synthesis through cPGES or mPGES-2 (Figure **63**) or from non-enzymatic isomerisation of PGH_2. Interference of 3α-OH-8,24-dien-TA with spontaneous decay of PGH_2 appears improbable. Thus, the additional inhibition of PGE_2 synthesis most likely arises from interference with other PGE_2 synthases. Interestingly, this property got lost in case of the 3β-hydroxy-derivative. DH-NA and the RAs (except of DH-k-RA) barely inhibited mPGES-1 activity although they represent *seco*-derivatives of the effective BAs. Obviously, the tight attachment of the carboxylic function to the A ring of BAs is essential for interference with mPGES-1, while the interaction of the *seco*-derivatives is reduced due to the positioning of the carboxylic group on a highly flexible tether. Notably, in contrast to DH-RA, the 11-keto-analogue DH-k-RA was highly effective, whereas the 11-keto-derivatives of BAs rather showed lower efficacy [511]. LAs inhibited mPGES-1 activity as well, with decisively increased potency of Ac-OH-LA compared to analogues that are not hydroxylated in position C-28.

In LPS-primed monocytes, the effectiveness of several triterpenic acids on PGE_2 formation was hampered. The 3-OH-TAs and 3-oxo-TA inhibited PGE_2 formation but the assessment of mPGES-1 inhibition by Ac-TAs, Ac-OH-LA and DH-k-RA was not feasible due to the induction of AA release (see chapter 4.2) or due to shunting mechanisms. As monocytes are capable to synthesize a broad array of eicosanoids [586], shunting of PGH_2 to PGE_2 synthesis may results from inhibition of other AA- or PGH_2-metabolizing enzymes. For example, extensive shunting was also observed with the TX synthase inhibitor CV4151, which increased the PGE_2 levels about

fivefold. Similarly, β-BA (30 µM) did not suppress PGE_2 formation in stimulated monocytes, although it was reported to be a potent inhibitor of mPGES-1-mediated PGE_2 synthesis in IL-1β-treated A549 cells [511].

Also in LPS-stimulated human whole blood, 3β-OH-TA and 3-oxo-TA potently suppressed mPGES-1-mediated PGE_2 synthesis, whereas the Ac-TAs were markedly less effective. In contrast to monocytes, DH-k-RA and Ac-OH-LA inhibited PGE_2 formation in whole blood, whereas the 3α-OH-TAs were inactive. The proposed shunt mechanism that was assumed to be responsible for the failure of DH-k-RA and Ac-OH-LA in monocytes, appears to be less relevant in this assay, which could result from the small number of monocytes in whole blood and a differential behaviour in other cell types. The failure of 3α-OH-TAs to inhibit PGE_2 formation in the whole blood assay might be due to concomitant induction of AA supply e.g. from platelets (see chapter 4.2) but remarkably, the 6-keto $PGF_{1\alpha}$ levels were not increased. Also the extracts from *Boswellia* species inhibited PGE_2 formation in whole blood, and again the a. f. from *B. papyrifera* was the most potent among all fractions. At higher concentrations, inhibition by this extract was hampered, which once again can be attributed to shunting of substrate or to AA mobilization through TAs.

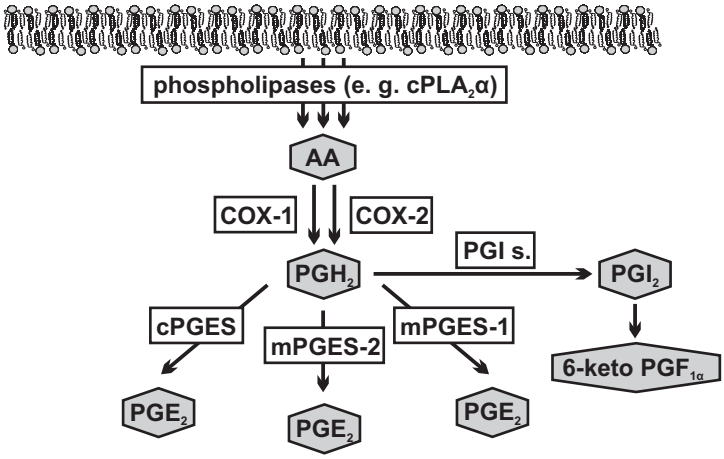


Figure 63: Biosynthesis of PGE_2.

To assess the selectivity of triterpenic acids that effectively suppressed mPGES-1 activity, their potency on further enzymes that contribute to inflammatory PGE_2 formation was analyzed (see Figure **63**). As shown in chapter 4.1, Ac-OH-LA potently suppresses $cPLA_2\alpha$-driven AA release in cell-free and cellular assays. Although the acid fraction from *B. papyrifera*, 3-oxo-TA and 3α-Ac-8,24-dien-TA significantly suppressed cell-free $cPLA_2\alpha$ activity, inhibition did not exceed 40%, even at high concentrations (30 µM or 30 µg/ml). This action may be related to unspecific membrane effects. Furthermore, TAs failed to suppress or rather stimulated AA release in a cellular context as discussed in chapter 5.2. On the contrary, DH-k-RA only suppressed AA

release in the cellular model, whereas it barely affected the isolated enzyme. Apparently, inhibition of AA release by DH-k-RA is due to interference with structures that regulate AA release rather than to direct interaction with cPLA$_2$α itself. Similarly, the a. f. from *B. papyrifera* and *B. serrata* suppressed AA release in stimulated platelets at 3 µg/ml, although they were barely effective in the cell-free assay at this concentration. Again, the suppressive effect was not observed at higher concentrations, which is presumably due to the stimulatory effects of TAs.

Considerable interaction with COX-1 activity was found for TAs, Ac-OH-LA and DH-k-RA in AA-stimulated platelets and direct inhibition of COX-1 was confirmed in a cell-free assay, though at higher concentrations. The reduction of 12-HHT formation in A23187-stimulated whole blood may be attributed to inhibition of COX-1 activity as well, since COX-2 is barely expressed in naïve blood. Impaired 12-HHT formation in platelets and whole blood might result also from interaction with intracellular signalling pathways such as p38 MAPK activation (see chapter 4.2.8). This would provide an explanation for the limited concentration-dependency of 12-HHT inhibition by TAs in whole blood and the minor efficacy in the cell-free assay. Although the test compounds moderately inhibited COX-2 in a cell-free system, no effect was found in a cellular model at concentrations up to 10 µM. Also in LPS-stimulated whole blood, where PGH_2 is supplied by COX-2 [615], the COX-derived 6-keto $PGF_{1\alpha}$ was not impaired by the test compounds. The interference of TAs, DH-k-RA and Ac-OH-LA with COX-1 potentially could mediate additional (adverse) effects as observed for NSAIDs, but mPGES-1 is suppressed at markedly lower concentrations. Thus, the effect on mPGES-1 is physiologically more relevant, especially in consideration of the plasma levels of the triterpenic acids.

3-oxo-TA and the acid fraction of the extract from *B. papyrifera* showed significant anti-inflammatory effects *in vivo* in a model of carrageenan-induced pleurisy in rats, as measured by reduction of inflammatory cells in the pleural cavity, reduced exudate volume and impaired levels of PGE_2, 6-keto $PGF_{1\alpha}$ and LTB_4. However, these effects cannot exclusively be ascribed to inhibition of mPGES-1 since one would expect that reduced PGE_2 levels are accompanied by constant 6-keto $PGF_{1\alpha}$ levels. In contrast, all the tested parameters were reduced to a similar extent, which suggests that a common target may mediate the main effects in this model (e.g. inhibition of the invasion of inflammatory cells by interference with chemotaxis or extravasation). For instance, BAs were shown to interact with cathepsin G and thereby reduced chemoinvasion of PMNL *in vitro* [544], and TAs also inhibit cathepsin G with similar potency (see chapter 4.5.1). 3-oxo-TA and the acid fraction of *B. papyrifera* extract were less effective than β-BA, which inhibits PGE_2 formation in the same model by about 50% [511]. Reduced efficacy resulting from stimulatory effects on AA release appears unlikely since 3-oxo-TA did not induce such effects in

human whole blood and also because this would rather lead to an increase in 6-keto $PGF_{1\alpha}$. Possibly, the applied model does not evidently reflect an impact on mPGES-1. In fact, mPGES-1 is only incompletely induced within 4 h after application of carrageenan [616], whereas cPGES and mPGES-2 are constitutively expressed in pleural leukocytes [617]. Therefore PGE_2 synthesis in this first phase of pleurisy is only partly formed by mPGES-1 and the effects that can be achieved by exclusive blockade of this enzyme are limited. As demonstrated in mPGES-1 knockout models, mPGES-1 plays a crucial role in *in vivo* models of chronic inflammation such as collagen-antibody-induced arthritis [322]. The assessment of mPGES-1 inhibitors in similar models promises results that are more distinct. The relatively small effect of the extract is in line with the results found for β-BA, since the administered dose corresponds to about 0.45 mg/kg β-BA, which only led to moderate effects *in vivo* [511].

In summary, a broad variety of triterpenic acids from *Boswellia* species was investigated with respect to the novel target structure mPGES-1 and several compounds were found to inhibit mPGES-1 activity. Effectiveness was preserved in cellular models and in whole blood, though some compounds (Ac-TAs, DH-k-RA and Ac-OH-LA) were found to interact with additional targets in AA metabolism, leading to interferences in particular assays. Regarding the AA-PGH_2-PGE_2 cascade, mPGES-1 was inhibited with IC_{50} values of 0.4 to 3 µM for the TAs, DH-k-RA and Ac-OH-LA, whereas COX and $cPLA_2\alpha$ were only suppressed with IC_{50} values of 10 µM or higher. Thus, under appropriate dosage these compounds promise selective inhibition of PGE_2 synthesis through mPGES-1, without affecting prostanoids that maintain physiologic functions. Though the pharmacokinetics of the investigated compounds are unknown, the IC_{50} values for inhibition of mPGES-1 are low and thus, required plasma levels might be reasonably achieved by standard dosage. For instance, the contents of TAs in extracts from *B. papyrifera* and *B. serrata* are almost comparable to those of BAs [498]. And for BAs, which possess similar structures as the analyzed compounds, steady-state plasma levels ranged from 0.1 µM for AKBA to 10 µM for β-BA after treatment with an extract from *B. serrata* in established dosage [563].

5.4 Impact of triterpenic acids and extracts from *Boswellia* species on 5-LO

5-LO represents the first identified target for BAs and for a long time its inhibition was accepted as the principal molecular mode of action of frankincense formulations [512]. Today this opinion is debated and recent work has shown that 5-LO might be not of pharmacological relevance as target because of the unfavourable pharmacokinetic properties of BAs (i.e. high albumin binding), the marginal content of AKBA (the most potent 5-LO inhibitor) in extracts from *B. serrata* and the

loss of efficiency in whole blood [603]. Moreover, additional molecular targets were found to be inhibited by compounds that are more abundant in frankincense formulations or with lower IC_{50} values (see chapter 2.5.4.3). However, the evaluation of 5-LO inhibition by extracts from different *Boswellia* species and by triterpenic acids besides BAs remained fragmentary so far. This study demonstrates the inhibition of purified 5-LO by all acid fractions of extracts from *Boswellia* species tested, whereas the neutral compounds from *B. carteri* showed no significant inhibition. Although Indian frankincense (*B. serrata*) was originally associated with this target [530], the effect on 5-LO was even more distinct for the extract from *B. papyrifera*. This observation is not surprising, considering the relatively high amount of the most potent BA (AKBA) in this extract (13.1%) compared to minor contents in the extract from *B. serrata* (2.2%) [498]. In fact, inhibition by AKBA alone could account for the effect exerted by the extract from *B. papyrifera*; a concentration of 10 µg/ml extract corresponds to about 2.5 µM AKBA, which matches its IC_{50} value of 2.9 µM in this test system [603]. On the other hand, the calculated AKBA concentration in 10 µg/ml of extract from *B. serrata* is only 0.4 µM, therefore AKBA can only partially contribute to the potent 5-LO inhibition exerted by this extract. As KBA also represents a minor compound in this extract and the IC_{50} values of the more abundant 11-methylene-BAs even exceed 30 µM, the contribution of other compounds to 5-LO inhibition appears plausible. The testing of diverse triterpenic acids revealed that some of them were similarly effective 5-LO inhibitors like AKBA or KBA. As all these triterpenic acids represent lipophilic acids and BAs were found to inhibit 5-LO through competition with AA at an allosteric binding site [534], an analogue mechanism may be assumed for the other triterpenic acids as well. As stated for BAs [533], the most potent inhibitors feature an additional hydrophilic function at a certain distance from the carboxylic moiety. 3-oxo-TA and 3β-OH-TA carry the 3-oxo- or 3β-hydroxy-moiety, respectively; DH-k-RA in analogy to KBA possesses the 11-keto-function and Ac-OH-LA presents the C-28-hydroxy-group. The corresponding analogues, which lack these functions or offer acetylated hydroxy-functions, are significantly less active. When the additional hydrophilic function is situated closely to the carboxylic group, the inhibitory potential decreases, as can be seen for β-BA and LA, and it is further diminished in the acetylated analogues again (e.g. in A-BA and Ac-LA). Interestingly, the 3α-OH-8,24-dien-TA is markedly less active than its 3β-hydroxy-analogue, and the flexion of the ring system resulting from displacement of the double bond from position 8 to position 7 even decreases the inhibitory effect. Presumably, the orientation of both hydrophilic groups to the same surface of the allosteric binding site is a prerequisite for effective inhibition. Ineffectiveness or incomplete inhibition of the enzyme by triterpenic acids that do not fit in this pattern does not implicate that these compounds do not bind 5-LO; as shown for the non-polar

amyrin, such substances still compete for the same binding site but simply do not exert intrinsic inhibitory activity [533].

In the cellular model of A23187- plus AA-stimulated PMNL, the effects of the triterpenic acids largely corresponded to those in the cell-free assay. It is noteworthy that the **inhibitory effects** of 3α-Ac-8,24-dien-TA and 3-oxo-TA were not impaired in comparison to the cell-free assay. In a previous study, these TAs were shown to induce MEK activation in A23187-stimulated PMNL, which counteracted the inhibitory effects and led to increased 5-LO activity instead [538]. These **stimulatory effects** were supposed to result from ERK-mediated phosphorylation and activation of 5-LO (Figure **64**). Alternatively, activation of MEK could mediate amplified AA release through ERK-mediated phosphorylation of $cPLA_2\alpha$ [174] and thereby enhance 5-LO translocation and activation [229]. This is supported by the data in this work, since the stimulatory effects of TAs on 5-LO product formation vanished upon stimulation in the presence of exogenous AA and the inhibitory effects (resulting from direct inhibition of 5-LO) prevailed. Furthermore, a stimulatory effect on 5-LO activity through MEK/ERK-mediated phosphorylation of 5-LO is improbable in A23187-stimulated PMNL, but rather appears at low Ca^{2+} levels [380].

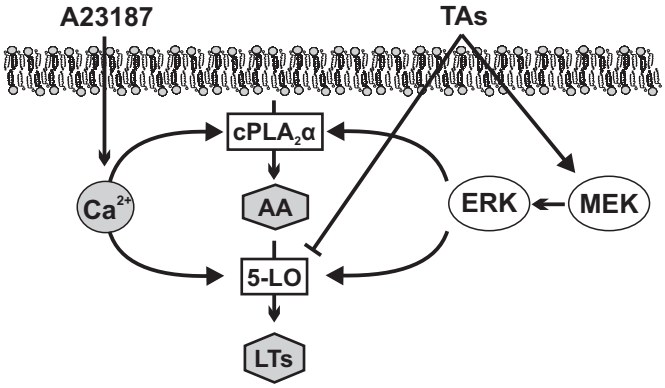


Figure 64: Regulation of 5-lipoxygenase product formation in stimulated PMNL by tirucallic acids.

Interestingly, the 3-OH-TAs and DH-k-RA showed relatively low IC_{50} values. Given the complex regulation of 5-LO in a cellular context (e.g. by the cellular redox tone, protein phosphorylation etc., see chapter 2.2.5.3) further investigation is needed to disclose the underlying mechanisms of this additional inhibitory effect.

As discussed for BAs [603], the relevance of the 5-LO-inhibitory effect of triterpenic acids in a pathophysiological context remains questionable. Like BAs, the most potent 5-LO inhibitors in this study also failed to suppress 5-LO product formation in a whole blood assay. As demonstrated for BAs, this is presumably due to the high affinity of triterpenic acids to plasma proteins, which prevails over the affinity to 5-LO. Actually, many pathological states that are successfully treated

with frankincense formulations lack a controlling role of LTs, which rather discloses a crucial role of 5-LO inhibition in the anti-inflammatory effects of frankincense [603, 618]. However, the herein presented triterpenic acids from frankincense extracts are potent direct inhibitors of 5-LO, most likely acting on the same allosteric binding site of the enzyme as BAs. The structure-activity relationship that was proposed for BAs can be expanded to the presented compounds and provides further insight into the structure of the allosteric AA binding site of 5-LO.

5.5 Impact of triterpenic acids and frankincense extracts on neutrophil proteases CG and HLE

The proteases CG, HLE and proteinase 3 are highly expressed in neutrophils and released in substantial amounts from azurophil granules upon stimulation e.g. with TNFα, LPS, PAF, PMA or fMLP [619]. Neutrophil proteases mediate a plethora of processes, which promote the inflammatory reaction in terms of cell infiltration, cytokine signalling, cell activation and apoptosis. But they also induce tissue injury, especially under chronic inflammatory conditions [437]. Thus, inhibition of CG or HLE was suggested in the therapy of rheumatoid arthritis, bronchial asthma and psoriasis [619-621], and all of these diseases are traditionally treated with frankincense formulations. Inhibition of HLE [542] and CG activity [544] by BAs was shown in previous studies.

Direct binding of BAs to CG was demonstrated causing potent inhibition of the proteolytic activity with IC_{50} values in the submicromolar range [544]. BAs also suppressed functional effects of CG, such as chemoinvasion of neutrophils in matrigel and CG-mediated Ca^{2+} mobilization in platelets. Reduced CG activity in whole blood from patients treated with a frankincense extract supports the physiologic relevance of this interaction. Here, we assessed the inhibitory potential of further triterpenic acids from *Boswellia* species on CG and HLE and compared them with selected BAs. Even though the CG assays were performed according to the protocol described in literature [544], the inhibitory effect of β-BA was more than tenfold lower than reported. The control inhibitor in contrast functioned properly and even exceeded the effect demonstrated in literature. Deviation in the specific activity of the enzyme preparations may partially explain these differences. Almost all tested compounds effectively suppressed the enzyme activity, with 3-oxo-TA, 3α-OH-7,24-dien-TA, DH-k-RA, DH-NA, LA and especially Ac-LA being even more potent than β-BA or A-BA. In an automated docking approach, BAs were demonstrated to bind to the active site cleft of CG [544]. In this model, the A, B and C rings are located on the protein surface and thereby allow some structural variation; hence, the binding of the BAs occurs with similar affinity, reflected by similar IC_{50} values. The *seco*-derivatives of BAs (RAs and NAs) exhibited similar

potency compared to β-BA, underlining the tolerance towards modifications in the A ring. LAs are structurally related to BAs, with variations in the E ring. Although the D an E rings are situated in a lipophilic pocket, this alteration does not impair the affinity to CG, as long as the lipophilic character and the spatial dimensions are widely preserved. Hydroxylation of C-28 in Ac-OH-LA increases the hydrophilicity of the rings D and E, thereby disfavours binding and consequently reduces the inhibitory effect. Despite the structural diversity of TAs and BAs, the former potently suppressed CG activity as well. The tested compounds had similar effects on purified enzyme or when the supernatant of stimulated PMNL was used as source of CG. The acid fractions from frankincense extracts also led to considerable inhibition of CG, whereas the neutral fraction isolated from *B. carteri* resin was ineffective, approving the necessity of an acidic function in inhibitors that mimic the control inhibitor JNJ-10311795 [544].

The inhibition of HLE by BAs was discovered in 1997 [542] and interaction with other triterpenic acids like ursolic acid was even found earlier [543]. Compared to ursolic acid, BAs are rather moderate inhibitors with an IC_{50} value of about 15 µM for AKBA. As already observed for CG, the inhibitory potential of test compounds fluctuates considerably depending on the assay conditions (specific activity of the enzyme). In this study, ursolic acid impaired HLE activity by about 55% at a concentration of 10 µM, although its IC_{50} value was reported to be 1 µM [542]. At 10 µM, AKBA did not appreciably influence HLE activity, which is in line with literature data. However, at 10 µM all tested compounds were equally or more potent than AKBA and some triterpenic acids (3α-Ac-8,24-dien-TA, RA and Ac-LA) inhibited HLE activity as potent as ursolic acid. The structural requirements for inhibition of HLE are obviously distinct from those found for CG. For ursolic acid, binding to the enzyme's binding pockets $S_{3\text{-}5}$ was suggested, with interactions of the carboxyl moiety to Arg^{217} in S_5 and further hydrophilic interactions of the 3-hydroxy-moiety and S_3 [543]. Analogue binding may be assumed for TAs since they present a similar distribution of the hydrophilic moieties, whereas BAs, RAs/NAs and LAs would require another orientation of their basal scaffold to allow an interaction of their carboxylic group with Arg^{217}. In contrast to the interaction with CG, an acidic function is not obligatory for inhibition of HLE. Lipophilic triterpenes such as amyrin or lupeol were also reported to inhibit HLE activity [542, 619]. This was also reflected by the efficacy of the neutral fraction from the *B. carteri* extract, which displayed equal potency like the acid fraction of the same extract. The acid fractions from *B. papyrifera* and *B. sacra* were the most potent extracts, but even at concentrations of 100 µg/ml, their inhibitory effect did not attain the potency of the recognized HLE inhibitor sivelestat.

Collectively, neutrophil proteases are targets of several triterpenic acids isolated from frankincense and inhibition of these enzymes may be relevant for the anti-inflammatory effects of frankincense formulations. At least regarding CG, the potency of BAs leads to functional cellular effects, which may likewise be achieved by other potent triterpenic acids. Clinically, the oral administration of frankincense extracts provides plasma levels of inhibitory triterpenes that are adequate to reduce CG activity in the blood [544]. The experiments in this study demonstrate that several quantitatively important triterpenic acids from frankincense are equally or more potent inhibitors of CG and HLE than BAs, and thereby may relevantly contribute to the therapeutic effects of frankincense extracts.

5.6 Effects of triterpenic acids from *Boswellia* species on glucocorticoid receptor signalling

Both, glucocorticoids and frankincense formulations are used in the treatment of chronic inflammatory diseases. Structurally, triterpenic acids (especially TAs) present similarities with glucocorticoids (Figure **65**). On the other hand, the tirucallane-, lupane-, oleanane- and ursane-scaffolds of the *Boswellia* triterpenes possess different configurations as compared to the conserved pregnane-scaffold of glucocorticoids. Also the different safety profiles of frankincense formulations (see chapter 2.5.4.2) and glucocorticoids (e.g. regarding the formation of oedema, hypertension and hypokaliaemia) do not support the interference of compounds from frankincense with glucocorticoid signalling.

The hypothesis that frankincense-derived triterpenic acids may act on the classical glucocorticoid-receptor-mediated transcription of glucocorticoid-responsive genes was assessed in this study. Since the genomic effects of glucocorticoids are relatively protracted and the tested compounds interact with several signalling pathways, a dual luciferase reporter approach in combination with the parallel assessment of a non-inducible dual luciferase approach was utilized to exclude unspecific biases resulting from varying transfection efficiency and effects on cell viability. In fact, some of the tested compounds mediated cell death in the utilized cancer cell line A549. For TAs, the induction of apoptosis of prostate cancer cell lines through inhibition of Akt was recently reported [557]. Multiple targets including topoisomerases, NF-κB and MAPK signalling were proposed as triggers of BA-induced apoptosis in numerous cancer cell lines (reviewed in [512]). However, for all of the tested compounds, cell viability was sufficient for proper translation of the luciferases. The calculation of the relative luciferase activity effectively corrected errors arising from such additional interferences. Although the compounds were tested in relatively high concentrations, none of them induced the reporter gene. Obviously, the structural analogy of the

triterpenic acids and glucocorticoids did not suffice to fulfil the stringent structural requirements of glucocorticoid receptor ligands. However, this finding does not exclude a possible interference of triterpenic acids from frankincense on non-genomic effects of glucocorticoids, which are also accepted as major mechanisms for the anti-inflammatory activity of glucocorticoids (though still poorly understood [481]). Furthermore, enzymes that are involved in the metabolism of glucocorticoids may represent possible indirect targets affecting glucocorticoid signalling/effects. For instance, glycyrrhetinic acid, the triterpenic acid aglycone of glycyrrhizin from liquorice (*Glycyrrhiza glabra*) was found to inhibit several steroid-metabolizing enzymes (e.g. 5β-reductase and 3α-, 3β- and 11β-hydroxysteroid dehydrogenases) and provokes the accumulation of active endogenous cortisol and the reduction of inflammation [622-624].

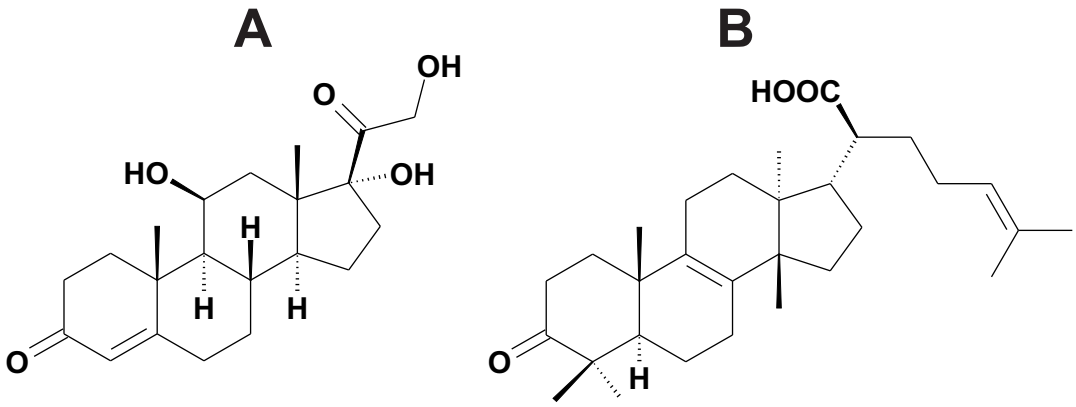


Figure 65: Structure of cortisol (A) and 3-oxo-tirucallic acid (B).

6 Summary

Herbal remedies have made a decisive contribution to the development of modern treatment of diseases. Not only because they were the only drugs available in ancient medicines and still are in a multitude of cultures, but also since they led to the discovery of numerous pharmacological targets and served as lead structures for a plenty of synthetic drugs. Frankincense formulations, originating from the oleo-gum resin of *Boswellia* spec., are traditionally used in the treatment of various diseases with a primarily inflammatory background. Their beneficial effect in the treatment of inflammatory bowel diseases, osteoarthritis, bronchial asthma and cancer was confirmed in several clinical pilot studies but the principal mechanisms leading to this efficacy are still matter of investigation. As frankincense comprises substantial amounts of the genus-specific boswellic acids (BAs), these compounds were considered to be responsible for the pharmacologic effects and were included in the quest for molecular targets. Diverse target structures of BAs have been identified so far: 5-lipoxygenase (5-LO), platelet-type 12-lipoxygenase (p12-LO), cyclooxygenase-1 (COX-1), microsomal prostaglandin E_2 (PGE_2) synthase-1 (mPGES-1), cathepsin G (CG), human leukocyte elastase (HLE), IκB kinase and topoisomerases. The contribution of some of these BA-target-interactions to anti-inflammatory activity is debatable, as the plasma levels of the proposed bioactive BA derivatives after administration of conventional doses of frankincense extracts are too low to affect them. However, outstanding potency of the abundant BAs lacking the 11-keto moiety on mPGES-1 and CG activity suggests major roles of these targets, which is supported by *in vivo* data. Apart from BAs, investigation of other compounds in the oleo-gum resin was rather neglected. Thus, the resin comprises appreciable amounts of structurally diverse triterpenic acids and a vast plethora of neutral compounds. Actually, BAs only make up about 10% of frankincense resin. Comprehensive consideration of all major compounds in the respective test systems is essential for the assessment of different *Boswellia* species and extraction techniques with the objective to yield frankincense formulations with optimized properties.

In this thesis, frankincense extracts from different *Boswellia* species and a variety of triterpenic acids isolated thereof were investigated in assays on 5-LO, COX-1 and -2, mPGES-1, HLE and CG activity and the impact of these compounds on the respective targets was compared to BAs. Moreover, the inhibitory and stimulatory effects of triterpenic acids on a novel target, the cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$), were investigated.

In a cell-free assay, 3-*O*-acetyl-28-hydroxy-lupeolic acid (Ac-OH-LA) concentration-dependently inhibited cPLA$_2$α activity, whereas diverse BAs, tirucallic acids (TAs), roburic/nyctanthic acids (RAs/NAs) and other lupeolic acids (LAs) failed in this respect. Obviously, the C-28-hydroxy-moiety is crucial for the inhibitory activity. Ac-OH-LA exhibited an IC_{50} value of 3 µM. Also in A23187-stimulated platelets, monocytes and PMNL, Ac-OH-LA diminished arachidonic acid (AA) release in the low micromolar range. AA metabolite formation in these cells was reduced as well and with similar potency. In contrast, upon stimulation with exogenous AA, the inhibitory effect was markedly attenuated or totally abolished. The cPLA$_2$α-dependent aggregation of platelets upon stimulation with collagen was suppressed by Ac-OH-LA, whereas no effect was seen after cPLA$_2$α-independent stimulation with U46619. Unfortunately, in a test system using human whole blood to mimic more physiological conditions, inhibition of cPLA$_2$α was hampered, most likely resulting from the high affinity of Ac-OH-LA to plasma proteins. After all, inhibition of cPLA$_2$α by Ac-OH-LA will hardly contribute to the anti-inflammatory effects of frankincense formulations since Ac-OH-LA is a minor constituent of the resin and its affinity to the enzyme is possibly not sufficient to overcome affine plasma protein binding. However, Ac-OH-LA could represent a valuable lead structure for the development of simplified and safe cPLA$_2$α inhibitors with improved bioavailability and lower toxicity compared to established cPLA$_2$α inhibitors.

Besides BAs, the TAs represent an abundant group of triterpenic acids in the resin from *B. papyrifera* and *B. serrata*. Here, TAs were found to induce AA release in platelets but not in neutrophils and this effect did not require the presence of Ca^{2+}. As expected, the formation of the AA metabolites 12(S)-hydroxy-5-cis-8,10-trans-heptadecatrienoic acid (12-HHT) and 12(S)-hydroxy-10-trans-5,8,14-cis-eicosatetraenoic acid (12-HETE) in platelets was significantly increased at TA concentrations above 3 to 10 µM, and 12-HETE synthesis was continuously elevated for at least 60 min. TA-induced metabolite production was largely suppressed by the selective cPLA$_2$α inhibitor RSC-3388, and TAs induced cPLA$_2$α translocation to the membranous compartment of platelets, which commonly correlates with cPLA$_2$α activity. Stimulation of cPLA$_2$α activity by TAs turned out to be induced by Ca^{2+} mobilization and the activation of p38 mitogen activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) signalling pathways, whereas no stimulating effect was seen in cell-free assays. Especially in Ca^{2+}-depleted cells, TAs led to phosphorylation of cPLA$_2$α at Ser^{505}, which most likely was mediated by p38 or JNK. Moreover, Ca^{2+}/calmodulin-modulated protein kinase II (CaMKII) inhibitors were found to inhibit TA-induced 12-HHT and 12-HETE formation. Together, TAs stimulate cPLA$_2$α activity through different pathways in platelets: Ca^{2+} mobilization predominates in a Ca^{2+}-containing environment,

whereas cPLA$_2$α phosphorylation by p38 or JNK prevails after deprivation of Ca^{2+}. Activation via CaMKII seems to promote cPLA$_2$α activity independently of the presence of Ca^{2+}.

Recently, BAs were identified as potent inhibitors of PGE_2 synthesis. Inhibition of mPGES-1 as the fundamental principle represents a smart approach to reduction of pro-inflammatory PGE_2: The production of further prostanoids is barely affected and thus, typical side effects of COX inhibitors are largely avoided. In a screening of different extracts from *Boswellia* species on mPGES-1 activity in a cell-free assay, the acid fraction from *B. papyrifera* was the most potent fraction and 3-oxo-8,24-dien-TA (3-oxo-TA) was revealed as most potent ingredient. The analysis of different triterpenic acids yielded various TAs, as well as 4(23)-dihydro-11-keto-RA (DH-k-RA) and Ac-OH-LA as potent inhibitors of mPGES-1 (IC_{50} = 0.4 - 3 µM). Thus, their potency is superior to that of BAs (IC_{50} > 3 µM). Using lipopolysaccharide (LPS)-primed monocytes as cellular test system for mPGES-1 activity, 3-*O*-acetyl-TAs (Ac-TAs), DH-k-RA and Ac-OH-LA failed to inhibit PGE_2 synthesis, presumably due to induction of AA release (see previous paragraph) or to shunting of substrate (AA or PGH_2) resulting from interaction with further AA- or PGH_2- metabolizing enzymes. 3-OH-TAs and 3-oxo-TA effectively suppressed PGE_2 formation comparably to the selective mPGES-1 inhibitor MD-52. In human whole blood, 3-β-hydroxy-8,24-dien-TA (3β-OH-TA), 3-oxo-TA, DH-k-RA and Ac-OH-LA inhibited LPS-stimulated PGE_2 synthesis without notably affecting other COX products (6-keto $PGF_{1\alpha}$); the 3α-OH-TAs and 3-Ac-TAs were inefficient in this test system. Experiments evaluating the selectivity towards further enzymes participating in PGE_2 synthesis showed that cPLA$_2$α, COX-1 and -2 were only inhibited by markedly higher concentrations of the triterpenic acids than mPGES-1. Furthermore, the effects on these enzymes were hampered in whole blood assays. For *in vivo* evaluation, 3-oxo-TA and the acid fraction of the extract from *B. papyrifera* were analyzed in a rat pleurisy model. Both treatments led to moderate inhibition of inflammation with significant reduction of inflammatory cells in the pleural cavity. Interestingly, the suppression of the PGE_2 levels was paralleled by reduced 6-keto $PGF_{1\alpha}$ levels. Thus, the anti-inflammatory effects cannot exclusively be attributed to inhibition of mPGES-1. In summary, triterpenic acids with diverse structural properties inhibit mPGES-1. In frankincense resin, some of these compounds, foremost 3-oxo-TA, are comprised in substantial amounts and show enhanced potency on mPGES-1 activity compared to BAs. Thus, considering the fact that inhibition of mPGES-1 is one of the most plausible rationales for the anti-inflammatory activity of frankincense extracts, these compounds may significantly contribute to the reduction of PGE_2 synthesis and the anti-inflammatory efficacy of frankincense.

BAs exhibit a unique mode of action mediating inhibition of 5-LO. Even though inhibition of this target was recognized as the active principle of frankincense for a long time, more recent data negate a significant contribution to clinical anti-inflammatory effects. Anyway, other triterpenic acids were barely examined in terms of interaction with 5-LO. In a cell-free test system, several triterpenic acids inhibited 5-LO activity with some of them being as effective as the most potent BAs. Conspicuously, compounds that exhibit an additional hydrophilic moiety with the same orientation as the carboxylic group were the most potent inhibitors. The acid fractions of extracts from *Boswellia* species potently inhibited 5-LO activity, whereas the neutral fraction of an extract from *B. carteri* was barely active. The extract from *B. papyrifera* was most potent, which correlates with relatively high levels of 3-*O*-acetyl-11-keto-β-BA (AKBA) and 3-oxo-TA. In neutrophils, the inhibitory effects on 5-LO activity were preserved or were even increased in case of the 3-OH-TAs and DH-k-RA. As already found for BAs, the inhibition of 5-LO by TAs, DH-k-RA and LAs was hampered in a whole blood assay, which might be due to the high affinity of triterpenic acids to plasma proteins. Thus, 5-LO inhibition presumably does not significantly contribute to the clinical anti-inflammatory effects of frankincense, neither through BAs nor through other triterpenic acids.

The neutrophil proteases HLE and CG are recognized targets of BAs. Functional cellular effects of CG inhibition by BAs and reduced CG activity in blood from patients that were treated with frankincense extracts suggest clinical relevance of this interaction. Several triterpenic acids besides BAs potently inhibited the activity of purified CG and of CG in a protein mixture excreted from stimulated neutrophils. The effects of 3-oxo-TA, 3α-OH-7,24-dien-TA, DH-k-RA, DH-NA, LA and especially 3-*O*-acetyl-LA (Ac-LA) were even more distinct than those of BAs. Obviously, the binding site on CG allows broad variation of the triterpenic scaffold, which is in line with results from automated docking studies with BAs. The most potent extract to inhibit CG was the acid fraction from *B. papyrifera* resin, whereas neutral compounds from *B. carteri* were ineffective.
Most of the tested triterpenic acids inhibited HLE activity more potent than BAs with 3α-Ac-8,24-dien-TA, RA and Ac-LA being the most effective compounds. The structural requirements for inhibition of HLE clearly differ from those of CG. Even the neutral fraction of the extract from *B. carteri* effectively inhibited HLE activity.
Collectively, the inhibition of neutrophil proteases may at least partly account for the activity of frankincense formulations in diverse inflammatory diseases. The data of this work suggest a major contribution of other triterpenic acids besides BAs to these effects.

Similarities of glucocorticoids and triterpenes from *Boswellia* species in terms of their structure and medical indication raised the question if triterpenes could act by interaction with the glucocorticoid receptor. Therefore, a luciferase reporter assay on glucocorticoid receptor response element activity was performed. Interestingly, none of the broad variety of triterpenes effectively stimulated the luciferase activity. Also the neutral fraction of the extract from *B. carteri* was ineffective. Thus, compounds from *Boswellia* species do not act directly on glucocorticoid signalling. This does not rule out interaction with the metabolism of endogenous cortisol or with non-genomic pathways of glucocorticoids, even though the lack of typical side effects after administration of frankincense formulations does not support such interactions.

Summing up, this work assesses the impact of numerous compounds isolated from *Boswellia* species on recognized targets of BAs, namely 5-LO, COX-1 and -2, mPGES-1, HLE and CG. This allows the evaluation of TAs, RAs/NAs and LAs with respect to their contribution to the overall anti-inflammatory effects of frankincense extracts. For all of the investigated target structures, the inhibitory potential of BAs was accomplished or even exceeded by several triterpenic acids. Especially TAs that make up substantial amounts in frankincense oleo-gum resins turned out to be potent inhibitors of mPGES-1 and CG. These are supposed to be the most relevant targets for the anti-inflammatory effects of frankincense formulations *in vivo*. The potent inhibition of mPGES-1 and CG by TAs widely prevailed over the stimulating properties on AA release at higher concentrations. Unfortunately, pharmacokinetic data that are necessary for conclusive evaluation only exist for BAs yet. Anyway, this work suggests a pivotal role of triterpenic acids besides BAs to the evident anti-inflammatory actions of frankincense extracts. Thus, extraction procedures should tend to include these compounds rather than avoiding them. The most potent extract in all of the investigated target structures in this work was the acid fraction from *B. papyrifera* oleo-gum resin. The efficacy correlates with high contents of AKBA and 3-oxo-TA. Finally, Ac-OH-LA was identified as novel inhibitor of cPLA$_2$α. Due to the minor content of Ac-OH-LA the inhibition of this additional target might not considerably contribute to the anti-inflammatory effects of frankincense extracts, but Ac-OH-LA may serve as lead structure in the quest for safe and bioavailable inhibitors of cPLA$_2$α.

7 Zusammenfassung

Pflanzliche Arzneistoffe haben die Entwicklung der heutigen Pharmakotherapie maßgeblich geprägt. Nicht nur weil sie früher die einzig verfügbaren Medikamente darstellten und heute in vielen Kulturkreisen immer noch darstellen, sondern auch weil sie zur Entdeckung zahlreicher pharmakologischer Zielstrukturen beitrugen und dadurch als Leitstrukturen für viele synthetische Arzneistoffe dienten. Weihrauchzubereitungen, gewonnen aus dem Harz von *Boswellia*-Arten, werden traditionell in der Behandlung von Krankheiten mit zumeist entzündlichem Hintergrund eingesetzt. Ihre positive Wirkung in der Therapie von entzündlichen Darmerkrankungen, Osteoarthritis, Asthma bronchiale und Krebserkrankungen wurde in mehreren klinischen Studien mit Pilotcharakter bestätigt. Dennoch sind die molekularen Hintergründe, welche zu dieser Wirksamkeit führen, immer noch Gegenstand der Forschung. Weihrauch enthält wesentliche Mengen an den artspezifischen Boswelliasäuren (BAs), weshalb diese Substanzen für die pharmakologischen Wirkungen verantwortlich gemacht wurden und auf der Suche nach molekularen Zielstrukturen als Basis dienten. Verschiedene Interaktionspartner wurden dabei identifiziert: 5-Lipoxygenase (5-LO), 12-Lipoxygenase (12-LO, Thrombozyten-Typ), Cyclooxygenase-1 (COX-1), mikrosomale Prostaglandin E_2 (PGE_2) Synthase-1 (mPGES-1), humane Leukozytenelastase (HLE), Cathepsin G (CG), IκB Kinase und Topoisomerasen. Für die Wechselwirkungen von BAs mit einigen dieser Zielstrukturen ist dabei fraglich, welchen Beitrag sie zur entzündungshemmenden Wirkung leisten, da die jeweils wirksamen BA-Derivate die benötigten Plasmaspiegel nach Gabe üblicher Weihrauchextraktdosen nicht erreichen. Eine Schlüsselrolle scheint hingegen die hochpotente Wirkung der häufigeren BA-Derivate ohne 11-keto-Gruppierung auf mPGES-1 und CG innezuhaben, die auch durch *in vivo* Studien bekräftigt wurde. Die Erforschung anderer Substanzen des Harzes neben BAs wurde in der Vergangenheit eher vernachlässigt. So enthält Weihrauch beachtliche Mengen an strukturell vielseitigen Triterpensäuren und eine gewaltige Fülle an Neutralbestandteilen. Tatsächlich machen BAs nur etwa 10% des Weihrauchharzes aus. Die umfassende Berücksichtigung aller Hauptbestandteile in den entsprechenden Testsystemen ist jedoch für die Bewertung verschiedener *Boswellia*-Arten und Extraktionstechniken notwendig, um Weihrauchpräparate mit optimierten Eigenschaften zu erhalten.

Im Rahmen dieser Arbeit wurden Weihrauchextrakte verschiedener *Boswellia*-Arten, sowie eine Reihe von daraus isolierten Triterpensäuren in Testsystemen auf 5-LO-, COX-1- und -2-, mPGES-1-, HLE- und CG-Aktivität untersucht und mit BAs verglichen. Außerdem wurden

hemmende sowie stimulierende Effekte von Triterpensäuren auf eine neuartige Zielstruktur, die zytosolische Phospholipase $A_2\alpha$ ($cPLA_2\alpha$), untersucht.

Im zellfreien System hemmte 3-*O*-Acetyl-28-Hydroxy-Lupansäure (Ac-OH-LA) konzentrationsabhängig die $cPLA_2\alpha$-Aktivität mit einem IC_{50}-Wert von 3 µM, wohingegen verschiedene BAs, Tirucallensäuren (TAs), Robur- und Nyctanthinsäuren (RAs/NAs), sowie in Position C-28 nicht hydroxylierte Lupansäuren (LAs) weitgehend inaktiv waren. Auch in A23187-stimulierten Thrombozyten, Monozyten und polymorphkernigen Leukozyten (PMNL) wurde die Arachidonsäure (AA)-Freisetzung durch Ac-OH-LA in Konzentrationen im unteren mikromolaren Bereich gehemmt. Die Bildung von AA-Metaboliten wurde mit vergleichbarer Potenz unterdrückt, wobei dieser Effekt durch Zugabe von exogener AA deutlich abgeschwächt wurde oder sogar vollständig verloren ging. Die $cPLA_2\alpha$-abhängige Thrombozytenaggregation nach Stimulation mit Kollagen wurde durch Ac-OH-LA gehemmt, wohingegen die $cPLA_2\alpha$-unabhängige Aggregation durch Stimulation mit U46619 nicht beeinflusst wurde. Leider ging die Hemmung der $cPLA_2\alpha$ im physiologischeren Vollblutmodell verloren, was vermutlich mit der hohen Affinität von Ac-OH-LA zu Plasmaproteinen erklärt werden kann. Letztendlich trägt die Hemmung der $cPLA_2\alpha$ durch Ac-OH-LA vermutlich kaum zur entzündungshemmenden Wirksamkeit von Weihrauchzubereitungen bei: Ac-OH-LA stellt einen Nebenbestandteil im Harz dar und seine Affinität zum Enzym reicht möglicherweise nicht aus, um die affine Plasmaproteinbindung zu überwinden. Hingegen könnte Ac-OH-LA eine wertvolle Leitstruktur in der Entwicklung sicherer und einfacher $cPLA_2\alpha$-Inhibitoren darstellen, die im Vergleich zu bisherigen Hemmstoffen eine verbesserte Bioverfügbarkeit bei geringer Toxizität versprechen.

Neben den BAs stellen TAs eine quantitativ bedeutsame Gruppe von Triterpensäuren in Harzen von *B. papyrifera* und *B. serrata* dar. In dieser Arbeit wurde gezeigt, dass TAs die AA-Freisetzung in Thrombozyten verursachen. Dieser Effekt war unabhängig von Ca^{2+} und war in neutrophilen Granulozyten nicht zu beobachten. Die Bildung der AA-Metaboliten 12(S)-Hydroxy-5-cis-8,10-trans-heptadecatriensäure (12-HHT) und 12(S)-Hydroxy-10-trans-5,8,14-cis-eicosatetraensäure (12-HETE) wurde ab TA-Konzentrationen von 3 bis 10 µM signifikant erhöht und dauerte im Falle von 12-HETE über mindestens 60 min an. Die TA-induzierte AA-Metabolitbildung wurde durch den selektiven $cPLA_2\alpha$-Inhibitor RSC-3388 weitgehend unterdrückt. Außerdem verursachten TAs die Translokation der $cPLA_2\alpha$ zu Membranstrukturen der Thrombozyten, was üblicherweise mit erhöhter $cPLA_2\alpha$-Aktivität einhergeht. Als Ursache für die Stimulation der $cPLA_2\alpha$-Aktivität durch TAs wurde die Mobilisierung von Ca^{2+}, sowie die Aktivierung von p38

Mitogen-aktivierte Proteinkinase (MAPK) und c-Jun N-terminale Kinase (JNK) Signalwegen erkannt, wohingegen keine stimulierenden Effekte in zellfreien Systemen gefunden wurden. Vor allem nach Chelatierung von Ca^{2+} wurde die $cPLA_2\alpha$ nach TA-Behandlung an Ser^{505} phosphoryliert, was vermutlich durch p38 oder JNK verursacht wurde. Darüber hinaus hemmten Inhibitoren der Ca^{2+}/Calmodulin-modulierten Proteinkinase II (CaMKII) die TA-induzierte 12-HHT- und 12-HETE-Bildung. Zusammengefasst stimulieren TAs die $cPLA_2\alpha$-Aktivität über verschiedene Wege: Ca^{2+}-Mobilisierung dominiert in Ca^{2+}-haltiger Umgebung, wohingegen die Phosphorylierung der $cPLA_2\alpha$ durch p38 oder JNK nach Ca^{2+}-Entzug zu überwiegen scheint. Die Aktivierung über CaMKII scheint unabhängig von Ca^{2+} die $cPLA_2\alpha$-Aktivität zu fördern.

Kürzlich wurden BAs als hochwirksame Hemmstoffe der PGE_2-Synthese entdeckt. Die zugrunde liegende Hemmung der mPGES-1 stellt einen viel versprechenden Ansatz in der Reduzierung von entzündungsförderndem PGE_2 dar: Die Biosynthese anderer Prostanoide wird kaum beeinflusst, wodurch die für COX-Inhibitoren typischen Nebenwirkungen weitgehend vermieden werden. Aus der Testung verschiedener Extrakte aus *Boswellia*-Arten auf die mPGES-1-Aktivität in einem zellfreien Testsystem resultierte die Säurefraktion aus *B. papyrifera* als wirksamste Fraktion, mit 3-Oxo-8,24-dien-TA (3-oxo-TA) als maßgeblich wirksamkeitsbestimmende Substanz. Die Untersuchung verschiedener Triterpensäuren stellte verschiedene TAs, sowie 4(23)-Dihydro-11-Keto-RA (DH-k-RA) und Ac-OH-LA als hochpotente Hemmstoffe der mPGES-1 heraus (IC_{50} = 0,4 - 3 µM). Somit ist ihre Wirksamkeit derjenigen der BAs teilweise deutlich überlegen (IC_{50} > 3 µM). In einem zellulären Testsystem auf mPGES-1-Aktivität unter Verwendung Lipopolysaccharid (LPS)-vorstimulierter Monozyten war keine Hemmung der PGE_2-Bildung durch 3-*O*-Acetyl-TAs (Ac-TAs), DH-k-RA und Ac-OH-LA zu verzeichnen. Dies kommt vermutlich durch AA-Freisetzung (s. vorhergehender Abschnitt) oder Überschießen von Substrat (AA oder PGH_2) als Folge der Hemmung von weiteren AA- oder PGH_2-metabolisierenden Enzymen zustande. 3-OH-TAs und 3-oxo-TA hemmten die PGE_2-Bildung mit vergleichbarem Effekt wie der selektive mPGES-1-Inhibitor MD-52. In humanem Vollblut hemmten 3-β-Hydroxy-8,24-dien-TA (3β-OH-TA), 3-oxo-TA, DH-k-RA und Ac-OH-LA die LPS-stimulierte PGE_2-Synthese, ohne dass andere COX-Produkte sichtbar beeinflusst wurden (6-Keto-$PGF_{1\alpha}$). Die 3α-OH-TAs und Ac-TAs waren in diesem Testsystem inaktiv. Die Untersuchung der Selektivität gegenüber weiteren in die PGE_2-Biosynthese verwickelten Enzymen zeigte, dass $cPLA_2\alpha$, COX-1 und -2 erst bei deutlich höheren Triterpensäurekonzentrationen gehemmt wurden als die mPGES-1. Außerdem gingen die Effekte im Vollblutmodell verloren. 3-oxo-TA sowie die Säurefraktion des *B. papyrifera*-Extraktes wurden *in vivo* in einem Brustfellentzündungsmodell in

Ratten getestet. Beide Behandlungen bewirkten eine moderate Hemmung der Entzündung mit signifikantem Rückgang der Entzündungszellen im Pleuraspalt. Interessanterweise gingen die herabgesetzten PGE_2-Spiegel mit reduzierten 6-Keto-$PGF_{1\alpha}$-Mengen einher. Deshalb können die entzündungshemmenden Effekte nicht allein einer Hemmung der mPGES-1 zugeschrieben werden. Zusammenfassend konnten mehrere strukturell unterschiedliche Triterpensäuren als Hemmstoffe der mPGES-1 identifiziert werden. In Weihrauch kommen einige dieser Substanzen – allen voran 3-oxo-TA – in beachtlichen Mengen vor, wobei sie im Vergleich zu BAs eine stärkere Potenz auf die mPGES-1 aufweisen. Da die Hemmung der mPGES-1 einer der plausibelsten molekularen Wirkmechanismen für die Entzündungshemmung durch Weihrauchextrakte ist, tragen diese Substanzen vermutlich maßgeblich zur Reduktion der PGE_2-Bildung und damit zur anti-entzündlichen Wirksamkeit von Weihrauch bei.

BAs weisen einen einzigartigen Mechanismus in der Hemmung der 5-LO auf. Obwohl die Hemmung dieser Zielstruktur lange Zeit als wirksamkeitsbestimmendes Prinzip von Weihrauch galt, ist nach neueren Studien eine bedeutsame Beteiligung an den klinischen entzündungshemmenden Eigenschaften zweifelhaft. Weitere Triterpensäuren wurden im Hinblick auf ihre Wechselwirkungen mit der 5-LO bisher kaum untersucht. Im zellfreien Testsystem hemmten mehrere Triterpensäuren die 5-LO-Aktivität, wobei manche so wirksam waren, wie die potentesten BAs. Auffälligerweise waren diejenigen Substanzen, die eine weitere hydrophile Gruppe mit ähnlicher Orientierung wie die Carboxylgruppe aufweisen, die wirksamsten Hemmstoffe. Auch die Säurefraktionen von Extrakten verschiedener *Boswellia*-Arten hemmten die 5-LO-Aktivität deutlich, wohingegen die Neutralbestandteile eines Extraktes aus *B. carteri* kaum wirksam waren. Der Extrakt aus *B. papyrifera* war am wirksamsten, was mit dem hohen Gehalt an 3-*O*-Acetyl-11-keto-β-BA (AKBA) und 3-oxo-TA korreliert. In neutrophilen Granulozyten blieb die Hemmung der 5-LO erhalten oder verstärkte sich im Falle der 3-OH-TAs und DH-k-RA sogar. Wie dies auch schon für BAs festgestellt wurde, ging die Hemmwirkung der TAs, LAs und DH-k-RA auf die 5-LO im Vollblutmodell verloren, was vermutlich auf die hohe Affinität der Triterpensäuren zu Plasmaproteinen zurückzuführen ist. Damit trägt die Hemmung der 5-LO durch BAs oder andere Triterpensäuren vermutlich nicht merklich zur klinischen entzündungshemmenden Wirkung des Weihrauchs bei.

Die Proteasen HLE und CG aus neutrophilen Granulozyten sind anerkannte Zielstrukturen von BAs. Funktionelle zelluläre Effekte der CG-Hemmung durch BAs und eine herabgesetzte CG-Aktivität im Blut Weihrauchextrakt-behandelter Patienten messen dieser Wechselwirkung

klinische Bedeutung bei. Neben BAs konnten verschiedene Triterpensäuren die Aktivität von aufgereinigtem CG oder CG aus einer Proteinmischung aus degranulierten Neutrophilen hemmen. Die Effekte von 3-oxo-TA, 3α-OH-7,24-dien-TA, DH-k-RA, DH-NA, LA und vor allem 3-*O*-Acetyl-LA (Ac-LA) überstiegen sogar die der BAs. Offensichtlich lässt die Bindungsstelle an CG großzügige Veränderungen des Triterpengrundgerüstes zu, was mit den Daten aus Docking-Studien mit BAs im Einklang steht. Als wirksamster Extrakt auf die CG-Aktivität stellte sich die Säurefraktion des Harzes aus *B. papyrifera* heraus, wohingegen die Neutralbestandteile aus *B. carteri* wirkungslos waren.
Die meisten getesteten Triterpensäuren hemmten die HLE-Aktivität mit höherer Potenz als BAs, wobei 3α-Ac-8,24-dien-TA, RA und Ac-LA die wirksamsten Substanzen darstellten. Die strukturellen Voraussetzungen für die Hemmung der HLE unterscheiden sich deutlich von denen für CG. Sogar die Neutralbestandteile des Extraktes aus *B. carteri* hemmten die HLE-Aktivität.
Die Hemmung von Proteasen aus neutrophilen Granulozyten scheint zumindest teilweise für die Wirksamkeit von Weihrauchzubereitungen bei verschiedenen entzündlichen Erkrankungen verantwortlich zu sein. Die Ergebnisse dieser Arbeit legen eine maßgebliche Beteiligung von weiteren Triterpensäuren neben BAs nahe.

Die Ähnlichkeit von Glucocorticoiden und Triterpenen aus *Boswellia*-Arten, bezüglich ihrer Struktur sowie ihrer klinischen Anwendung, warf die Frage auf, ob Triterpene durch Interaktion mit dem Glucocorticoid-Rezeptor wirken könnten. Deshalb wurde im Rahmen dieser Arbeit ein Luciferase-Reporterassay auf die Aktivität von Glucocorticoid-Rezeptor-responsiven Elementen durchgeführt. Allerdings zeigte keines der Triterpene eine stimulierende Wirkung auf die Luciferase-Aktivität. Auch für den gesamten Neutralbestandteil-Extrakt aus *B. carteri* konnten keine Effekte verzeichnet werden. Damit wirken Substanzen aus *Boswellia*-Arten nicht direkt auf den klassischen Glucocorticoid-Signalweg. Dies schließt allerdings Wechselwirkungen mit dem Metabolismus endogenen Cortisols oder mit nicht-genomischen Signalwegen nicht aus. Jedoch liegen solcherlei Wechselwirkungen auf Grund des Ausbleibens typischer Nebenwirkungen nach der Behandlung mit Weihrauchzubereitungen nicht nahe.

Diese Arbeit behandelt die Beeinflussung von anerkannten Zielstrukturen der BAs (5-LO, COX-1 und -2, mPGES-1, HLE und CG) durch zahlreiche Inhaltsstoffe aus *Boswellia*-Arten. Dies erlaubt die Bewertung von TAs, RAs/NAs und LAs im Hinblick auf ihren Beitrag zur entzündungshemmenden Gesamtwirkung von Weihrauchextrakten. Die Wirksamkeit der BAs wurde für alle der untersuchten Zielstrukturen durch mehrere weitere Triterpensäuren erreicht oder

sogar übertroffen. Insbesondere TAs, welche beachtliche Anteile des Weihrauchharzes ausmachen, erwiesen sich als potente Hemmstoffe der mPGES-1 und des CG. Diese Zielstrukturen scheinen *in vivo* die größte Relevanz für die entzündungshemmenden Wirkungen von Weihrauchzubereitungen zu haben. Die potente Hemmung der mPGES-1 und des CG durch TAs überstieg dabei weitestgehend die stimulierenden Effekte auf die AA-Freisetzung, die erst bei höheren Konzentrationen auftraten. Leider wurden die für eine abschließende Beurteilung notwendigen pharmakokinetischen Daten bislang nur für BAs bestimmt. Dennoch legen die Erkenntnisse dieser Arbeit nahe, dass die Triterpensäuren neben BAs eine wesentliche Rolle in den anti-entzündlichen Wirkungen von Weihrauchextrakten spielen. Extraktionsverfahren sollten somit möglichst darauf abzielen, diese Substanzen zu erfassen anstatt sie auszuschließen. Der wirksamste Extrakt in allen hier behandelten Testsystemen war die Säurefraktion des Harzes aus *B. papyrifera*. Diese Wirksamkeit geht mit einem hohen Gehalt an AKBA und 3-oxo-TA einher. Ferner wurde Ac-OH-LA als neuartiger Hemmstoff der $cPLA_2\alpha$ identifiziert. Aufgrund des geringen Gehalts an Ac-OH-LA mag die Hemmung dieser neuen Zielstruktur nicht merklich zur Entzündungshemmung durch Weihrauchextrakte beitragen, jedoch könnte Ac-OH-LA auf der Suche nach sicheren und bioverfügbaren Hemmstoffen der $cPLA_2\alpha$ als Leitstruktur dienen.

8 References

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9 Publications

9.1 Original Publications

KOEBERLE, A., BAUER, J., VERHOFF, M., HOFFMANN, M., NORTHOFF, H. & WERZ, O. 2009. Green tea epigallocatechin-3-gallate inhibits microsomal prostaglandin E(2) synthase-1. *Biochem Biophys Res Commun,* 388**,** 350-4.

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SIEMONEIT, U., KOEBERLE, A., ROSSI, A., DEHM, F., VERHOFF, M., RECKEL, S., MAIER, T. J., JAUCH, J., NORTHOFF, H., BERNHARD, F., DOETSCH, V., SAUTEBIN, L. & WERZ, O. 2011. Inhibition of microsomal prostaglandin E2 synthase-1 as a molecular basis for the anti-inflammatory actions of boswellic acids from frankincense. *Br J Pharmacol,* 162**,** 147-62.

VERHOFF, M., SEITZ, S., NORTHOFF, H., JAUCH, J., WERZ, O. A novel C(28)-hydroxylated lupeolic acid from frankincense suppresses the biosynthesis of eicosanoids through inhibition of cytosolic phospholipase A2. *Biochem Pharmacol,* submitted.

VERHOFF, M., SEITZ, S., PAUL, M., NORTHOFF, H., JAUCH, J., WERZ, O. Modulation of arachidonic acid mobilization in human platelets by tirucallic acids from *Boswellia* species. Manuscript in preparation.

VERHOFF, M., MUELLER, D., SEITZ, S., PAUL, M., NORTHOFF, H., JAUCH, J., WERZ, O. Interaction of extracts and triterpenic acids from *Boswellia* species with PGE2 biosynthesis. Manuscript in preparation.

VERHOFF, M., MUELLER, D., SEITZ, S., PAUL, M., NORTHOFF, H., JAUCH, J., WERZ, O. Triterpenic acids from *Boswellia* species as inhibitors of 5-Lipoxygenase and Cathepsin G. Manuscript in preparation.

9.2 Patents

WERZ, O., VERHOFF, M., HENKEL, A., JAUCH, J. & SEITZ, S. 2009. Verwendung einer Tirucallensäure, einer Lupansäure oder einer Robursäure als Arzneimittel. *Deutsches Patent- und Markenamt.* Patent No. DE102009004436A1.

9.3 Oral presentations

VERHOFF, M., MULLER, C. & WERZ, O. 2008. *Boswellia* preparations: Current market situation and clinical trials. *Weihrauch Symposium.* Tübingen, Germany. *Phytomedicine,* 15**,** 543-544.

VERHOFF, M., SEITZ, S., JAUCH, J. & WERZ, O. 2010. Lupeolic acid derivatives from *Boswellia* species as inhibitors of the cytosolic phospholipase A(2 alpha). *58th International Congress of the Society for Medicinal Plant and Natural Product Research.* Berlin, Germany. *Planta Med,* 76, 1181.

VERHOFF, M. & WERZ, O. 2008. Targets und Wirkmechanismen von Boswelliasäuren. *PZ Fachexkursion.* Salalah, Sultanate of Oman.

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9.4 Poster presentations

VERHOFF, M., SEITZ, S., JAUCH, J. & WERZ, O. 2009. Tirucallic acids from *Boswellia* extracts as inhibitors of mPGES-1. *Jahrestagung der Deutschen Pharmazeutischen Gesellschaft.* Jena, Germany.

10 Acknowledgements

An erster Stelle möchte ich mich bei Herrn Prof. Dr. Oliver Werz bedanken, für das reizvolle Thema meiner Doktorarbeit, seine absolute Unterstützung und seinen allzeit verfügbaren, wertvollen Ratschlag.

Bei Herrn Prof. Dr. Stefan Laufer bedanke ich mich für die Übernahmen des Zweitgutachtens und für die Unterstützung des in Tübingen verbliebenen Teils des AK Werz.

Prof. Dr. Johann Jauch, Dr. Stefanie Seitz und Michael Paul danke ich für die erfolgreiche Kooperation, die fortlaufende Versorgung mit isolierten Harzkomponenten und die fruchtbaren, oftmals ins Philosophische gehenden Diskussionen.

Prof. Dr. Lidia Sautebin, Dr. Antonietta Rossi und Friederike Dehm danke ich für die herzliche Aufnahme und Unterstützung während meines Aufenthalts in Neapel.

Für den Zugang zum Szintillationszähler sowie die für Abwechselung sorgenden Gespräche bedanke ich mich bei Prof. Dr. Joachim Schultz.

Bei Prof. Dr. Hartmut Morck möchte ich mich für die großartige Möglichkeit bedanken, dass ich an der Exkursion an den Ursprung des Weihrauchs (Oman) teilnehmen durfte.

Prof. Dr. Hinnak Northoff und den Mitarbeitern der Transfusionsmedizin am Uniklinikum Tübingen danke ich für die geduldige Bereitstellung von Vollblut und Leukozytenkonzentraten.

Der Firma Aureliasan GmbH, insbesondere der Familie Ertelt, danke ich für die vielschichtige Unterstützung, die Bereitstellung aller erdenklichen Extrakte sowie das stetige Interesse an den laufenden Forschungsaktivitäten.

Beim gesamten Arbeitskreis Werz möchte ich mich für die hervorragende Arbeitsatmosphäre, den offenen Umgang und die allgegenwärtige Hilfsbereitschaft bedanken.
Dabei danke ich im Speziellen …
… Hanne Braun, dem guten Geist des Arbeitskreises, für die geduldige Hilfe bei allen erdenklichen organisatorischen Belangen.
… Daniela Müller für ihren großartigen Einsatz bei verschiedensten Testungen, für ihren Überblick bei der Verwaltung der Extrakt-Massen sowie allgemein für ihre geduldige und ausgleichende Art.

… Bianca Jazzar für den regen Austausch von Meinungen/Informationen/Equipment, für die immer kurzweilige Zusammenarbeit (zu jeder Tages- und Nachtzeit) und für ihren maßgeblichen Beitrag zum guten Betriebsklima.

… Dr. Ulf Siemoneit, Dr. Ulrike Bühring, Jana Precht und PD Dr. Bernd Kammerer für die professionelle Organisation und unterhaltsame Zusammenarbeit im Arzneibuchpraktikum.

… Felix Behnke und Susann Luderer für die gemeinschaftliche Betreuung des Praktikums Instrumentelle Analytik.

… dem Weihrauch-Forscherkreis Dr. Arne Henkel und Dr. Ulf Siemoneit für die Einarbeitung in verschiedenste Labormethoden, rege Diskussionen und das gelegentliche Asyl im „Boswell-Lab“.

… Dr. Felix Behnke für die Hilfestellung bei den target fishing-Experimenten, die zahlreichen wertvollen Gespräche sowie seinen bedingungslosen Einsatz in der Erforschung Tockeneis- und Stickstoff-basierter Phänomene.

… Dr. Christine Greiner für ihre aufopferungsvolle Hilfsbereitschaft, ihre vermittelnde Rolle im Helge-Wendy-Labor und insgesamt für die schöne Zeit auf dem gemeinsamen Weg in Freiburg und Tübingen.

… Dr. Andreas Köberle für seine geduldige Einarbeitung in die Welt der PGE_2-Biosynthese sowie seine große Hilfe bei der Planung des GRE Reporter Gene Assays.

… Dr. Carlo Pergola für die Teilhabe an seinem umfangreichen Expertenwissen, seine wertvollen Ratschläge und Informationen, die mir auch beim Erstellen dieser Arbeit eine große Hilfe waren.

… Dr. Dagmar Blaesius für ihre ordnende Hand und die Versorgung mit jeder Menge gutem Kuchen.

… Friederike Dehm für die große Unterstützung bei den *in vivo*-Experimenten und für Ihre Qualitäten als Reiseführerin in und um Neapel.

… Anja Schaible für den ergiebigen Austausch in Sachen Phospholipasen.

… Katja Wiechmann für die sorgsame Pflege der Zellkultur-Einrichtungen.

… Julia Bauer für das „die Stellung halten“ als sich der AK Werz in Tübingen nach und nach verflüchtigte.

Zu guter Letzt möchte ich mich bei meiner Familie bedanken, für ihren Beistand und die fortlaufende Unterstützung. Besonders danke ich Elisabeth Reichle für ihr Verständnis, ihre Geduld und ihren Zuspruch während der Entstehung dieser Arbeit.

11 Akademische Lehrer

Meine akademischen Lehrer an der Albrecht-Ludwigs-Universität Freiburg waren:

Prof. Dr. Dr. K. Aktories (Pharmakologie und Toxikologie)

Prof. Dr. A. Bechthold (Pharmazeutische Biologie)

PD Dr. R. Eickhorn (Physiologie)

Prof. Dr. Dr. A. W. Frahm (Allg. und analytische Chemie)

Prof. Dr. L. Hein (Pharmakologie und Toxikologie)

Prof. Dr. G. Herten (Physik)

Prof. Dr. R. Jackisch (Pharmakologie und Toxikologie)

Prof. Dr. M. Jung (Pharmazeutische und Medizinische Chemie)

Prof. Dr. A. Kaiser (Pharmazeutische Chemie und Chemische Nomenklatur)

Prof. Dr. M. Kist (Mikrobiologie)

Prof. Dr. N. Klugbauer (Pharmakologie und Toxikologie)

Prof. Dr. I. Merfort (Pharmazeutische Biologie)

Prof. Dr. D. K. Meyer (Pharmakologie und Toxikologie)

Prof. Dr. M. Müller (Pharmazeutische und Medizinische Chemie)

Prof. Dr. M. Plehn (Terminologie, Pharmaziegeschichte)

Prof. Dr. R. Schubert (Pharmazeutische Technologie)

Prof. Dr. K. Starke (Pharmakologie und Toxikologie)

Prof. Dr. R. Süss (Pharmazeutische Technologie)

Prof. Dr. B. Szabo (Pharmakologie und Toxikologie)

Prof. Dr. B. Wünsch (Quantitative Analytik)

Prof. Dr. E. Urban (Stereochemie)