Evaluation of the Anti-Inflammatory Effects of Novel Pirinixic Acid Derivatives

Evaluierung der entzündungshemmenden Wirkungen neuartiger Pirinixinsäurederivate

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1. Abbreviations

AA	arachidonic acid
AB	antibody
Ada	adenosine deaminase3
AKBA	3-O-acetyl-11-keto-boswellic acid
ALP	alkaline phosphatase
AP	activator protein
APS	ammonium persulfate
ATLs	aspirin-triggered lipoxins
ATP	adenosine triphosphate
ATR-FTIR	attenuated total reflection Fourier transform infrared
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumine
cAMP	cyclic adenosine monophosphate
C/EBP beta	CCAAT/enhancer binding protein beta
CLP	coactosin-like protein
COX	cyclooxygenase
$cPLA_2$	cytosolic phospholipase A2
CYP450	cytochrome P450
DAG	diacylglyceride
DHET	dihydroxyeicosatrienoic acid
DMEM	Dulbecco's modified Eagle's medium
DMF	dimethylformamide
DMSO	dimethyl sulfoxide

DPPH	diphenylpicrylhydrazyl
DTT	dithiothreitol
ECL	electrochemiluminescence
E. coli	*Escherichia coli*
EDTA	ethylenediamine-tetraacetic acid
EET	epoxyeicosatrienoic acid
EIA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FAD	flavin adenine dinucleotide
FCS	fetal calf serum
FLAP	5-lipoxygenase activating protein
fMLP	N-formyl-methionyl-leucyl-phenylalanine
GM-CSF	granulocyte/macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
GPx	glutathione peroxidases
Grb	growth factor receptor-bound protein
GSH	glutathione
HEDH	hydroxyeicosanoid dehydrogenase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HETE	hydroxyeicosatetraenoic acid
12-HHT	12-hydroxy-5,8,10-heptadecatrienoic acid
HpETE	hydroperoxy-6-trans-8,11,14-cis-eicosatetranoic acid
HPLC	high performance liquid chromatography
13-(S)-HpODE	13S-hydroperoxy-9Z,11E-octadecadienoic acid
IC_{50}	inhibiting concentration (50% of inhibition)

Ig	immunoglobuline
ICAM	inter-cellular adhesion molecule
IL	interleukin
IPTG	isopropyl-β-D-thio-galactoside
JNK	c-Jun NH2-terminal kinase
LB-medium	Luria Broth base - medium
LO	lipoxygenase
LOOH	lipid hydroperoxide
LPS	lipopolysaccharide
LT	leukotriene
LX	lipoxin
MAPEG	membrane-associated proteins in eicosanoid and glutathione metabolism
MAPK	mitogen-activated protein kinase
MBP	maltose-binding protein
MK	mitogen-activated protein kinase activated protein kinase
mPGES	microsomal prostaglandin E_2-synthase
MS	mass spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	sodium chloride
NBT	nitro-blue tetrazolium
NES	nuclear export sequence
NFAT	nuclear factor of activated T-cells
NLS	nuclear localization sequence
NP40	Nonidet P-40

NQO2	NAD(P)H dehydrogenase, quinone 2
NSAIDs	non-steroidal anti-inflammatory drugs
OAG	1-oleoyl-2-acetyl-sn-glycerol
PA	pirinixic acid
PAF	platelet activating factor
PAI	plasminogen activator inhibitor
PA-P	phosphatidic acid phosphatase
PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	prostaglandin
PGI	prostacyclin
PG buffer	PBS-glucose buffer
PGC buffer	PBS-glucose-Ca^{2+} buffer
PI	phosphatidylinositol
PK	protein kinase
PL	phospholipid
PMA	phorbol 12-myristate 13-acetate
PMNL	polymorphonuclear leukocytes
PMSF	phenylmethyl sulfonyl fluoride
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response element
PS	phosphatidylserine
ROS	reactive oxygen species
RT	room temperature

RXR	retinoid X receptor
SDS	sodium dodecyl sulfate
SDS-b	2× SDS loading buffer
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SH	Src homology
SPPARM	selective PPAR receptor modulator
SPR	surface plasmon resonance
STAT	signal transducer and activator of transcription
STI	soybean trypsin inhibitor
TFA	trifluoroacetic acid
THF	tetrahydrofurane
TG	thapsigargin
TGF	transforming growth factor
TNF	tumor necrosis factor
TRAP	TGFβ receptor-I-associated protein
TX	thromboxane
VCAM	vascular cell adhesion molecule
WB	Western Blot
w/o	without

2. Introduction

2.1. Inflammation

Acute inflammation is a beneficial host response to external or internal stimuli (e.g. infection, toxins, irritants or tissue injury) and is accompanied by the classic cardinal signs heat, redness, swelling and pain (**Fig. 1**) [1]. These manifestations reflect an increased regional blood flow caused by vasodilatation as well as an increase in microvascular permeability. In general, this process is self-limited due to endogenous anti-inflammatory and proresolving mediators (e.g. lipoxins, resolvins, protectins and maresins [2]), but if the host cannot manage to abrogate the inflammatory reaction, chronic conditions may arise, resulting in a loss of tissue or organ function. A variety of chronic diseases like asthma, rheumatoid arthritis, cardiovascular diseases, atherosclerosis, inflammatory bowel diseases, Alzheimer's disease and also cancer are associated with such an inappropriate inflammatory response [3], [4].

Fig. 1. **Cardinal signs of inflammation** (adopted from Lawrence, T. et al. Nat Rev Immunol, 2(10) 787-95, 2002). The characteristics of inflammation - heat, redness, swelling, pain, and loss of function (in case of a chronic state) - were defined by Celsus AD40.

Exposure to an inflammatory challenge leads to the release of early mediators such as complement factors C3a and C5a, vasoactive substances (histamine, bradykinin) and eicosanoids (prostaglandins (PGs) and leukotrienes (LTs)) within the injured tissue, promoting exudate formation and edema. Subsequently, proinflammatory cytokines (interleukin (IL)-1, tumor-necrosis factor (TNF), and chemokines like IL-8) are produced, inducing an upregulation of adhesion molecules (selectins, intercellular adhesion molecule (ICAM) 1 and vascular cell adhesion molecule (VCAM) 1) in the endothelium and enabling the recruitment of circulating neutrophils. These short-lived phagocytic cells clean the damaged tissue from cell debris, harmful material and microorganisms with the help of reactive oxygen species (ROS) and degradative enzymes [5]. In the course of inflammation, monocytes (which mature into macrophages) migrate to the respective tissue and ingest apoptotic neutrophils. Finally, anti-inflammatory mediators are released, and normal tissue function is restored.

2.2. Arachidonic acid cascade

As mentioned above, PGs and LTs are important signaling molecules in inflammation. Both classes of compounds are metabolites of all-*cis* 5,8,11,14-eicosatetraenoic acid (arachidonic acid, AA) liberated from the sn-2 position of membrane phospholipids by phospholipase A_2 (PLA_2) upon cellular activation by an appropriate stimulus. A variety of PLA_2 enzymes has been described based on primary structure, localization and Ca^{2+} requirement [6], but cytosolic $PLA_{2\alpha}$ ($cPLA_2$) is believed to be mainly responsible for the biosynthesis of eicosanoids [7].

Liberated AA can serve as a substrate for cyclooxygenases (COX-1 and COX-2) that produce PGs, thromboxanes (TX) and prostacyclin (PGI) - summarized as prostanoids. Alternatively, lipoxygenases (5-LO, 12-LO and 15-LO - their distinct actions are described in **2.3.**) catalyze the formation of LTs, lipoxins (LXs) and H(p)ETE, or cytochrome P450 (CYP450) enzymes convert AA into epoxyeicosatrienoic acid (EET) and dihydroxyeicosatrienoic acid (DHET) [8] (**Fig. 2** gives an overview of the AA cascade). The generated products excite distinct biological effects that are mediated via selective G protein-coupled receptors (GPCRs). Moreover, the short living AA can serve as a second messenger by affecting signaling pathways itself [9], forms isoprostane by reacting with free radicals, or is finally reincorporated into membrane phospholipids.

Prostanoids mediate fever, pain and inflammation, but they are also of importance in the regulation of the cardiovascular system, the gastrointestinal tract [10], renal functions,

angiogenesis and reproduction [11], [12]. The constitutively expressed COX-1 is supposed to be preferentially involved in physiological processes, whereas COX-2 is inducible and is found mainly in inflammatory cells in response to cytokine release [13]. The widely used non-steroidal anti-inflammatory drugs (NSAIDs) possess potent anti-inflammatory and analgetic properties, but also cause severe side-effects in the gastrointestinal tract [14] and in the kidney [15]. These complications have been ascribed to the suppression of physiologically relevant (COX-1 derived) prostanoids such as the cytoprotective PGE_2 and PGI_2 in the gastroduodenal epithelium. Selective COX-2 inhibitors, called coxibs, have been developed to overcome the gastrointestinal side-effects of traditional NSAIDs. However, in recent studies these drugs have been associated to an increased risk for cardiovascular events (e.g., myocardial infarction, stroke, systemic and pulmonary hypertension, congestive heart failure and sudden cardiac death [16]), and some of these drugs had to be withdrawn from market. The side effects of coxibs are thought to be related to an imbalance of anti-thrombotic and vasodilatory PGI_2 versus the prothrombotic TXA_2. To overcome the disadvantages of a complete suppression of PGs, selective inhibition of microsomal prostaglandin E_2 synthase (mPGES)-1 has been suggested [17], [18]. mPGES-1, which is functionally coupled to COX-2 [19], [20], is responsible for excessive PGE_2 synthesis upon cellular stimulation by IL-1β, TNFα and lipopolysaccharides (LPS), and seems to play an important role in pain, fever, inflammation, atherosclerosis and tumorigenesis [21], [22], [23].

EETs derived from CYP450 possess anti-inflammatory effects in the regulation of vascular functions [24], but they are also involved in endothelial cell proliferation and angiogenesis [25].

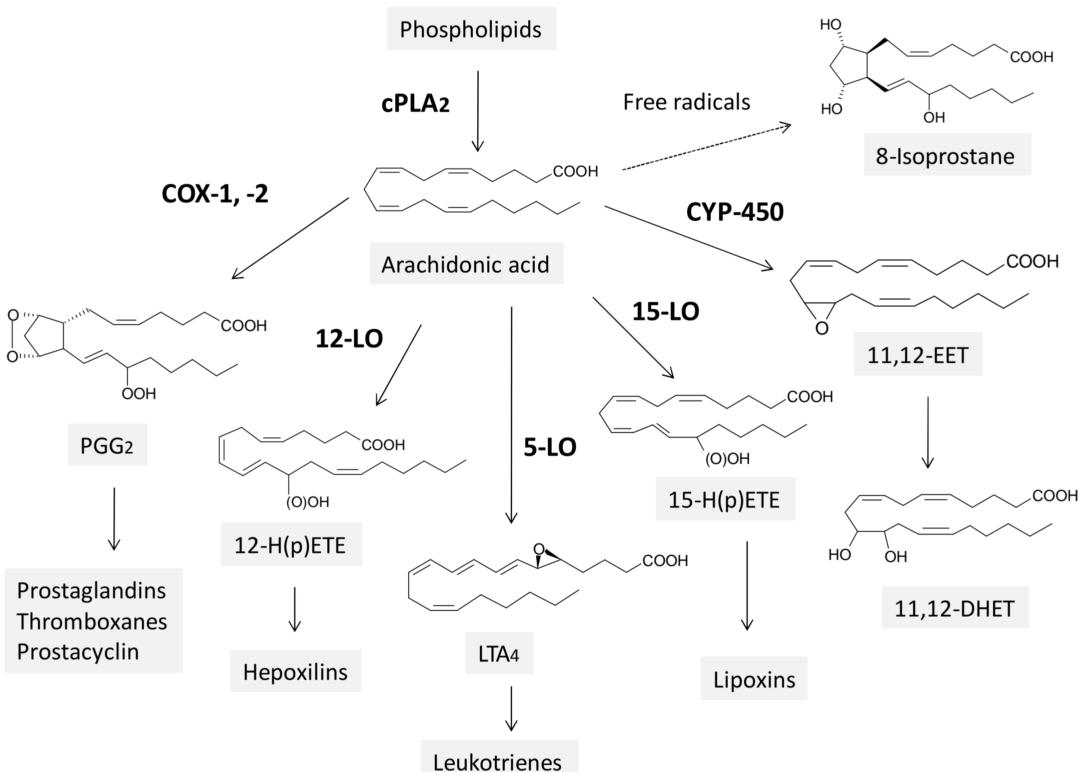


Fig. 2 Arachidonic acid (AA) cascade. AA is released from membrane phospholipids by cytosolic phospholipase A_2 ($cPLA_2$), and is further converted to prostanoids by cyclooxygenases (COX-1 and COX-2), to leukotrienes (LTs), lipoxins (LXs) and hydro(pero)xy-eicosatetranoic acid (H(p)ETE) by lipoxygenases (LOs), or to epoxyeicosatrienoic acid (EET) and dihydroxyeicosatrienoic acid (DHET) by cytochrome P450 (CYP450). Moreover, AA can react with free radicals to give isoprostane.

2.3. Lipoxygenases

LOs are non-heme iron-containing dioxygenases that catalyze the insertion of molecular oxygen into AA and other polyunsaturated fatty acids yielding distinct hydroperoxyeicosatetraenoic acids (HpETEs) which are then transformed into its corresponding alcohol (HETE). The following LOs are expressed in humans: 5(S)-LO, platelet-type p12(S)-LO, reticulocyte type 12/15(S)-LO I (in reticulocytes and eosinophils [26], [27]), 15(S)-LO II and epidermis type e12(R)-LO. The number in the name of the respective LO reflects the position at which oxygen is introduced (5-, 12-, 15-LO).

5-LO catalyzes the first two steps in the formation of LTs. These potent lipid mediators play crucial roles in the innate immune response and are involved in inflammatory diseases such as asthma, allergic rhinitis, inflammatory bowel disease, psoriasis and shock, but they have also been related to cardiovascular diseases and certain types of cancer (for review, see [28]).

12-LO derived 12-HpETE can be reduced to 12-HETE, or further metabolized to diHETEs and hepoxilins. 12-HETE promotes cell proliferation in cancer cells [29], cell motility [30], angiogenesis [31] and renal vasoconstriction. Increased levels of 12-HETE have been observed in patients with diabetes mellitus [32] and essential hypertension [33]. Hepoxilins induce chemotaxis of neutrophils and facilitate migration across intestinal epithelia [34].

15-HETE plays an important role in certain inflammatory diseases (e.g. proctocolitis) [35], but also certain anti-inflammatory actions of this 15-LO metabolite have been discussed. Thus, it decreases superoxide formation, exocytosis and migration of neutrophils. Moreover, it may block LTB_4 formation and induce apoptosis [36].

Lipoxins are formed through the concerted action of different LOs and, in case of aspirin-triggered lipoxins (ATLs), also of COX enzymes [37]. They belong to the pro-resolving lipid mediators that contribute to the resolution of an inflammatory reaction and are typically produced by transcellular biosynthesis (for review, see [38]).

2.3.1. 5-Lipoxygenase

5-LO was described for the first time in 1976 as the enzyme that catalyzes the formation of 5-HpETE and 5-HETE in rabbit polymorphonuclear leukocytes (PMNL) [39], and only three years later, the same group discovered 5-HETE and LTB_4 released from human leukocytes [40].

2.3.1.1. 5-LO reaction and leukotriene formation

5-LO catalyzes two distinct reactions: first, molecular oxygen is incorporated into AA (oxygenase activity), and subsequently, the LTA_4 synthase activity of the enzyme yields the 5,6 epoxide LTA_4 [41], [42] (**Fig. 3**). 5-HpETE is obtained by an abstraction of the pro-S hydrogen at C-7 of AA, followed by insertion of molecular oxygen at position C-5 [43]. In the next step, the pro-R hydrogen from C-10 of 5-HpETE is abstracted, and the radical is shifted to C-6 yielding LTA_4 [44]. Another metabolite of 5-HpETE is its corresponding alcohol 5-HETE which is the precursor of 5-oxo-ETE [45] and the lipoxins [46]. 5-oxo-ETE formation is catalyzed by the microsomal $NADP^+$-dependent enzyme 5-hydroxyeicosanoid dehydrogenase (5-HEDH), whereas lipoxins are generated by the combined action of 5-LO and 12-LO or 15-LO. The assay conditions such as the relative concentrations of free AA and 5-HpETE, membrane association, the amount of 5-LO, and the presence of 5-LO activating protein (FLAP) determine the ratio of LTA_4 to 5-HpETE [47], [48], [49], [50]. The unstable LTA_4 is either converted to LTB_4 by LTA_4-hydrolase or conjugated with reduced glutathione (GSH) yielding LTC_4 (mediated by LTC_4 synthase (LTC_4S)) [51]. Both LTB_4 and LTC_4 are released from the cell, and LTC_4 is hydrolyzed subsequently to LTD_4 which is then converted to LTE_4.

COOH
Arachidonic Acid
5-LO
(oxygenase)
OOH
COOH
5-HpETE
OH
COOH
5-HETE
5-LO
(LTA_4 synthase)
5-HEDH
O
COOH
O
COOH
5-oxo-ETE
LTA_4
LTA_4-hydrolase
LTC_4-synthase
OH
OH
COOH
LTB_4
OH
COOH
R
R = -Cys-Gly LTA_4
|
Glu
Glutamyl transpeptidase
R = -Cys-Gly LTD_4
Cysteinylglycinase
R = -Cys LTE_4

Fig. 3. **LT biosynthesis.** Free arachidonic acid (AA) is converted to the hydroperoxide 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetranoic acid (5-HpETE) by the oxygenase activity of 5-lipoxygenase (5-LO). Then, the LTA_4 synthase activity of 5-LO catalyzes the formation of the unstable epoxide LTA_4 that is either converted to LTB_4 by LTA_4 hydrolase, or conjugated with glutathione (GSH) to LTC_4 by LTC_4 synthase. LTC_4 is the precursor of the other cysteinyl-LTs D_4 and E_4. 5-HpETE can also be reduced to 5(S)-hydroxy-6-trans-8,11,14-cis-eicosatetranoic acid (5-HETE) and further oxidation by 5-hydroxyeicosanoid dehydrogenase (5-HEDH) leads 5-oxo-ETE.

The iron of 5-LO is in the ferrous state (Fe^{2+}) when the enzyme is inactive and is oxidized after treatment with lipid hydroperoxides (LOOHs) [52], [53]. Notably, hydrogen peroxide is not able to stimulate 5-LO. The course of the 5-LO reaction can be divided into three phases: i) an initiation phase where the iron is converted to the ferric form; ii) a linear propagation phase with a high turnover; and iii) a terminal phase of irreversible inactivation (**Fig. 4**). The rapid inactivation of 5-LO may be provoked by LOOHs generated during catalysis, although these molecules are important to initialize the enzyme reaction as well [54]. If glutathione peroxidases (GPx) are added to cell-free 5-LO activity assays, they inhibit product formation but also stabilize 5-LO because of their reducing effect on LOOHs [55], [28].

Reduction of 5-HpETE is thought to be mediated by a pseudoperoxidase activity of 5-LO [56], [57] which is stimulated by certain derivatives of diphenyl-N-hydroxyureas, 4-hydroxybenzofurans and 5-hydroxydihydrobenzofurans [58].

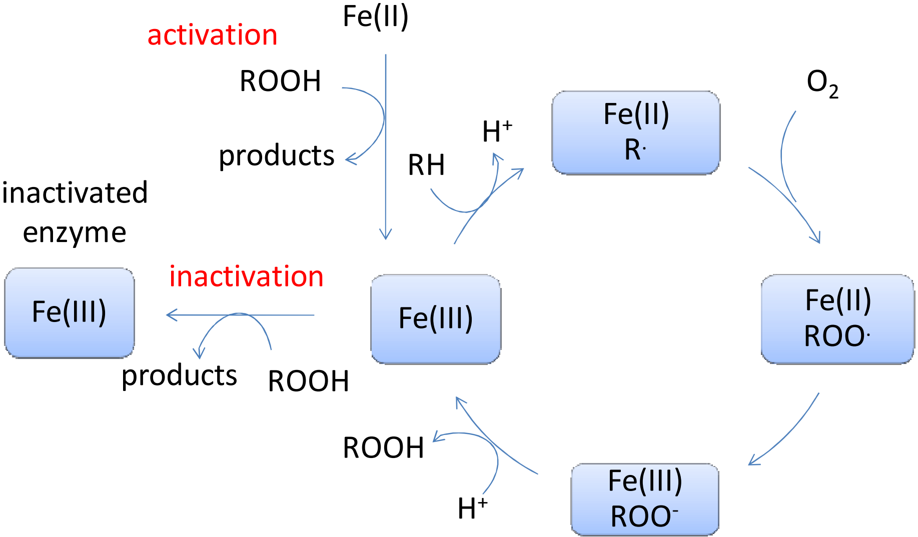


Fig. 4. **The catalytic cycle of 5-LO.** Activation of 5-LO requires oxidation of Fe^{2+} to Fe^{3+} mediated by lipid hydroperoxides (LOOHs), but LOOHs also terminate the catalytic cycle of the 5-LO reaction.

2.3.1.2. 5-LO gene and expression

The human 5-LO gene (ALOX5, 71.9 kbp) consists of 14 exons and is located on chromosome 10 [59]. Eight GC-boxes can be found in the promoter region (five of them are arranged in tandem), but no TATA and CAT boxes have been detected (**Fig. 5**). Although this pattern resembles typical housekeeping genes, 5-LO is expressed mainly in leukocytes, and suppression of its expression in other cell types is mediated by DNA methylation [60]. It has been discussed if variant DNA methylation may lead to an upregulation of 5-LO in epithelial tumor cells since the protein has been found there as well [61]. The transcription factors Sp1 and Egr-1 bind to the five tandem GC-boxes mentioned above, and for Sp1, a novel binding site upstream of the major transcription initiation site of the 5-LO gene was described in 2005 [62]. Mutations in the Sp1-binding sites provoke alterations in 5-LO expression, associated to physiological changes. Thus, carriers of ALOX5 variants are more susceptible to tuberculosis [63], and variations in the GC-boxes are related to atherosclerosis because of an increased intima-media thickness and an elevated plasma level of C-reactive protein [64].

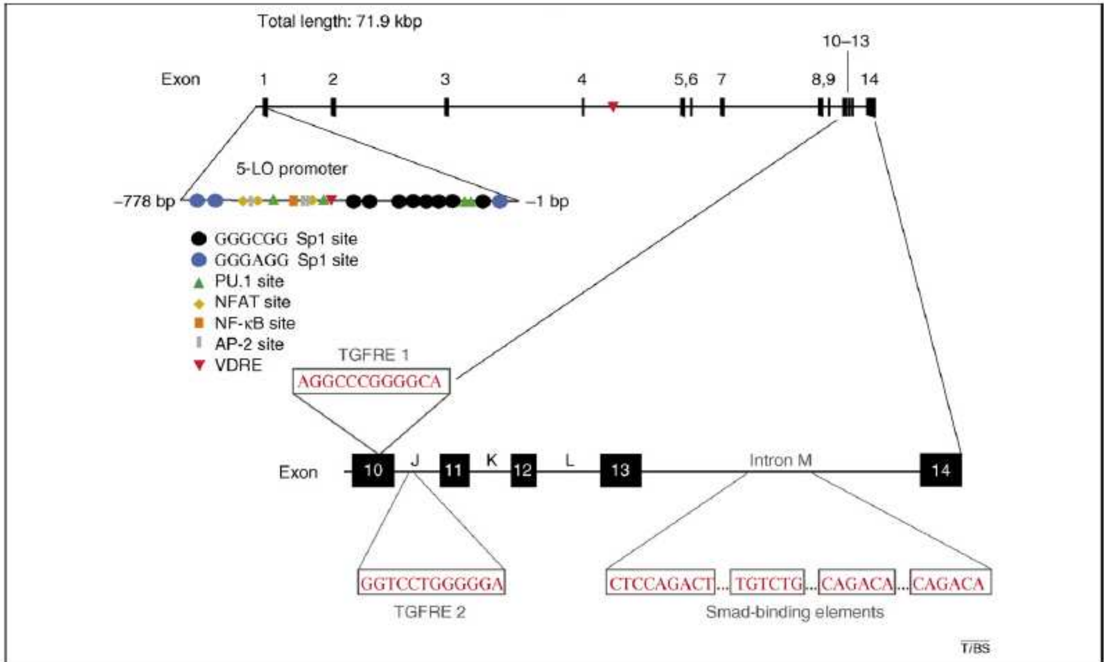


Fig. 5. **The human 5-LO gene.** The human 5-LO gene is located on chromosome 10, consists of 14 exons, and possesses several transcription initiation sites with the principal one (position 0) located 65 bp upstream of ATG. The regulatory sites of the gene are indicated in the figure. *Abbreviations*: TGFRE, TGF-β-response element. (from *Radmark, O. et al. Trends Biochem Sci 32, 332-41, 2007*)

5-LO expression occurs in granulocytes, monocytes/macrophages, mast cells, dentritic cells, mantle zone B lymphocytes (but hardly in germinal B cells and plasma cells) [65] and Langerhans cells as well as in fibroblasts in the skin, whereas the enzyme has not been found in platelets, endothelial cells and erythrocytes [66], [67]. In contrast, it has not been finally clarified yet if T lymphocytes express 5-LO. Moreover, 5-LO was detected in rat hippocampus and hippocampus of patients with Alzheimer's disease [68], [69]. Interestingly, differentiated myeloid cells and cell lines express significantly higher amounts of 5-LO as undifferentiated cells. This upregulation during leukocyte differentiation has been associated with certain inducers such as dimethyl sulfoxide (DMSO), retinoic acid, 1α,25-dihydroxyvitamin D3 [1,25$(OH)_2D_3$] and transforming growth factor *β* (TGF*β*) [70]. In addition, a stimulatory effect of granulocyte/macrophage colony-stimulating factor (GM-CSF) on 5-LO expression was observed in mature blood leukocytes. Interestingly, tissue macrophages produce higher amounts of 5-LO than monocytes in the blood [71], whereas if kept in culture, human monocytes lose 5-LO as well as FLAP over time [72], and additional growth factors are needed to maintain the expression.

2.3.1.3. 5-LO structure

Mammalian 5-LOs are monomeric, soluble proteins composed of 672 or 673 amino acids (for review, see [73]) resulting in a molecular mass of 72 to 80 kDa [74]. The amino acid sequence of human 5-LO was described already in 1988 [75] but the intrinsic instability of the enzyme hampered clarification of its three-dimensional structure for a long time, and 5-LO was only modeled based on the crystal structure of rabbit reticulocyte 15-LO (a 12/15-LO that shares 40 % sequence homology with 5-LO) [76], [77]. In 2011 Gilbert et. al. identified a 5-LO specific destabilizing sequence near the C terminus. By replacement of K653KK655 with ENL (the corresponding sequence from 8*R*-LO from *Plexaura homomalla*) together with exchange of several amino acids thought to be responsible for membrane-insertion as well as mutation of Cys240 and C561, stability of 5-LO could be strongly increased so that crystallization of the enzyme was possible [78]. 5-LO consists of two domains: an N-terminal C2-like ß-sandwich and an iron-containing catalytic domain at the C-terminus (residues 121-673) which is mainly helical in structure (**Fig. 6**). The non-heme catalytic iron is anchored by three histidine residues (His372, His367 and His550) and by the C-terminal Ile673. The active site is flanked by an arched helix as well as by helix α2 providing distinct residues which help to form an elongated cavity and prevent access to the catalytic iron. Besides several invariant residues which have also been

found in the active sites of other LOs [79], a number amino acids are specific for 5-LO, and some of them contribute to the formation of a “cork” which closes the catalytic cavity so that a conformational change is needed to allow access of the substrate [78].

5-LO activity can be influenced by phosphorylation on three serine residues in the catalytic domain: on Ser271 by mitogen-activated protein kinase (MAPK)-activated protein kinase (MK)-2/3 [80], on Ser663 by extracellular signal-regulated kinase (ERK)-2 [81] and on Ser523 by protein kinase (PK) A [82]. The β-sandwich of the smaller N-terminal domain (residues 1-114) possesses several ligand-binding loops [83]. Ca^{2+} has been shown to bind to Asn43, Asp44 and Glu46 within the C2-like domain, whereas other stimulatory factors of 5-LO, namely phosphatidylcholine (PC), glycerides (e.g. 1-oleoyl-2-acetyl-snglycerol, OAG) and coactosin-like protein (CLP) rely on three tryptophane residues (Trp13, 75 and 102).

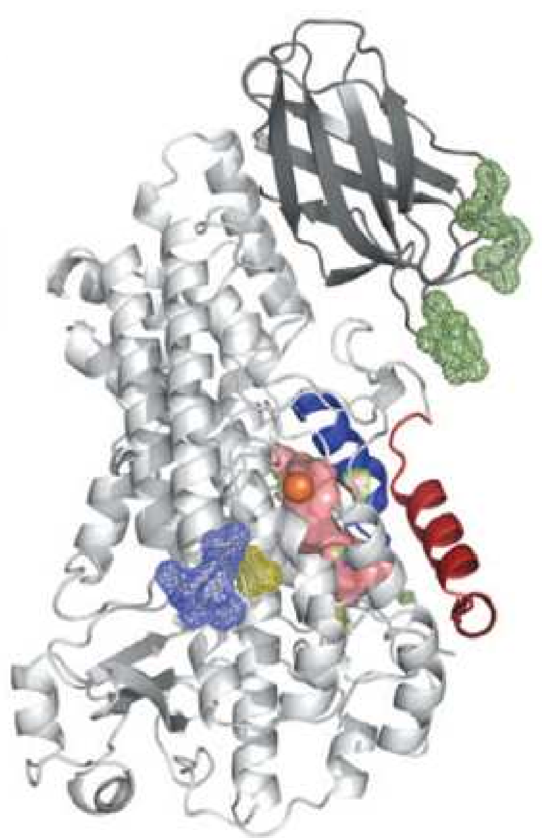


Fig. 6. Structure of human 5-LO. Structure of stable 5-LO as crystallized by Gilbert, N.C. et al.. The enzyme is composed of a C-terminal catalytic domain (residues 121-673; light gray) that is mainly helical, and an N-terminal C2-like β sandwich (dark gray). The putative membrane insertion residues mutated in stable 5-LO are shown in green. The active site is flanked by an arched helix (colored in blue) and by helix α2 (shown in red). The active site cavity is coloured in pink and the catalytic iron is presented as an orange sphere. The KKK→ENL substitution is indicated in mesh blue and the mutated proximal cysteines are colored in yellow (from *Gilbert, N.C. et al. Science 331(6014), 217-9, 2011*).

2.3.1.4. Regulation of 5-LO

5-LO catalysis is controlled in a complex manner and involves a variety of factors that influence the enzyme activity. Studies in cell free systems (isolated 5-LO, whole cell homogenates, subcellular fractions) have been useful to get an idea about the regulation of 5-LO activity. However, in intact cells 5-LO product formation is even more complicated than in cell-free systems. Thus, factors like subcellular localization of 5-LO, phosphorylation events and interaction with cellular proteins play an important role for the enzyme activity and have been subjected to multiple studies in the last decades (for review, see [66], [84]).

2.3.1.5. Factors determining 5-LO activity *in vitro*

Factors that stimulate 5-LO activity seem to facilitate binding of AA to the enzyme and to maintain the catalytic redox cycle. Most of these factors act at the N-terminal β sandwich (Ca^{2+}, PC, glycerides and CLP), whereas the binding site for adenosine triphosphate (ATP) has not been demonstrated yet (**Fig. 7**).

Calcium

Early studies on 5-LO purified from human leukocytes demonstrated that Ca^{2+} is able to induce the enzyme activity [85]. Full activation is reached at 4-10 µM Ca^{2+}, and the EC_{50} was determined between 1 and 2 µM. 5-LO binds Ca^{2+} (about two ions per 5-LO) in a reversible manner with a K_d of approximately 6 µM (for the native enzyme) [86], [87], and a similar dissociation constant (7-9 µM) was found for the His-tagged C2-like domain [88]. Notably, also Mg^{2+} (at millimolar concentrations present in cells) was shown to stimulate 5-LO activity in vitro [89]. However, neither Ca^{2+} nor Mg^{2+} is part of the catalytic mechanism, and 5-LO possesses basal activity also in absence of these cations. It was shown in mutagenesis studies that Asn43, Asp44 and Glu46 in the ligand binding loop 2 of the β sandwich are essential for activation of 5-LO by Ca^{2+} [83].

Stimulation of 5-LO by Ca^{2+} occurs only in presence of PC or CLP, and Ca^{2+} might be necessary for a 'productive' binding of these factors to the enzyme. Moreover, Ca^{2+} is needed for the interaction of 5-LO with membranes and stimulates its translocation to the nuclear envelope which is rich in PC [88]. It was found in a phase partition assay that Ca^{2+} increases the

hydrophobicity of 5-LO. Ca^{2+} binding also causes a higher affinity of 5-LO towards AA and thus provokes a change in the reaction kinetics resulting in substrate inhibition.

Finally, a low level of LOOHs is sufficient for 5-LO activation if Ca^{2+} is present, since inhibition of 5-LO by GPx is attenuated in this case [90]. In affinity labeling studies of rabbit 12/15-LO, an AA analog bound not only within the catalytic domain, but also to the N-terminal β sandwich [91]. This suggests that LOs might possess a second, regulatory fatty acid binding site, where also LOOHs could bind, possibly in a Ca^{2+} dependent manner.

Phosphatidylcholine

A common feature for C2 domains is their Ca^{2+}-induced association to membranes [92]. 5-LO activity is stimulated by microsomal membranes as well as by synthetic lipid vesicles that contain PC [85], [93]. Importantly, PC could not be replaced by phosphatidylserine (PS), phosphatidyl-ethanolamine (PE) or phosphatidylinositol (PI). Three Trp residues in the ligand binding loops of the β sandwich (Trp13, 75 and 102) are important for PC binding, and selectivity for this phospholipid is assumed to account for 5-LO translocation to the nucleus [88]. Pande et. al. demonstrated that also a cationic diacylglycerophospholipid is able to increase the membrane affinity of 5-LO, but it hardly stimulates the enzyme activity, possibly due to a 'non-productive' binding to the membrane [94]. This suggests that the proper orientation of 5-LO at the membrane might be extremely important for its catalytic activity. Moreover, it was shown by attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy that membrane binding of 5-LO leads to a dehydration of the membrane surface, and both the enzyme and lipids within the membrane gain stability upon their interaction. In subsequent studies, increases in the membrane fluidity were shown to facilitate 5-LO association, and it was proposed that this is the reason for a selective direction of 5-LO to the AA enriched nuclear envelope [95]. Notably, if a membrane preparation was supplemented with cholesterol, a decrease in 5-LO product formation was observed in cell free assays [95], and the same effect was shown in intact cells after treatment with cholesterol sulfate [96].

Glycerides

5-LO product formation in isolated human PMNL (supplemented with exogenous AA) can be stimulated upon addition of the cell-permeable OAG [97]. The intracellular Ca^{2+} level is not

increased under these conditions, and neither activation of MAPK nor translocation to the nuclear membrane is affected by OAG. Several glycerides were shown to activate 5-LO in a direct manner with OAG as the most effective compound [98]. Addition of Ca^{2+}, phospholipids or cellular membranes turned out to prevent the stimulatory effect of OAG. It was demonstrated that binding of glycerides is mediated by the same three Trp residues that are involved in PC association to 5-LO. Hence, a W13A/W75A/W102A triple mutant of 5-LO is not affected by OAG. In contrast to Ca^{2+}-induced PC binding, association of the uncharged glycerides to the C2-like domain of 5-LO may occur in absence of charge neutralization or changes in side chain orientations [88]. As observed for Ca^{2+}, OAG is able to protect 5-LO from inhibition by GPx [98].

Treatment of PMNL with 5-LO product synthesis-inducing stimuli such as Ca^{2+}-ionophore A23187, thapsigargin (TG) or chemokines leads to a formation of diacylglycerides (DAGs) [99], [100], [101], [102], [103], [104]. Pharmacological intervention with DAG generation via phospholipase D (PLD) and phosphatidic acid phosphatase (PA-P) in ionophore- or TG-activated PMNL causes a significant decrease of 5-LO translocation and product formation. The inhibitory effect can be reversed by addition of exogenous OAG. Therefore, it has been suggested that the formation of DAGs via the PLD/PA-P pathway after appropriate stimulation is one possible mechanism to activate cellular 5-LO.

Coactosin-like protein

In the course of a yeast two-hybrid assay, CLP was identified as a 5-LO binding protein [105]. Human CLP consists of 142 amino acids and shows similarity with the actin-binding coactosin of the slime mold *Dictyostelium discoideum*. An *in vitro* stoichiometry of 1:1 was determined for CLP binding to 5-LO, and interaction between the two proteins in intact cells was confirmed by co-immunoprecipitation experiments [106].

CLP can also bind F-actin (with a 1:2 stoichiometry), and it was found that two lysine residues (Lys75 and 131) close-by in the CLP sequence are necessary for binding to F-actin and to 5-LO, respectively [107]. Consequently, the binding sites overlap, and F-actin competes with 5-LO for association to CLP.

Ca^{2+}-induced 5-LO product formation is stimulated by CLP in the absence of PC, but this effect is increased upon simultaneous application with the phospholipid [108]. Similarly as membranes, CLP might act as a scaffold for 5-LO and increases its activity. Recently, the importance of 5-LO-Trp102 for binding to CLP was demonstrated by mutagenesis experiments [109]. Although CLP is not needed for Ca^{2+} to bind to 5-LO, activation of the enzyme only occurs in presence of Ca^{2+} [106], and the mode of binding is believed to be similar to the 'productive' or 'non-productive' type for membranes that was mentioned above.

It has been assumed that in intact cells, CLP is always bound to 5-LO, since both proteins are located in the cytosol in resting cells and translocate to the nuclear envelope upon activation by Ca^{2+} [108], [109]. Finally, it was suggested that simultaneous binding of 5-LO to CLP and the nuclear membrane may promote catalysis of the second step of LTA_4 synthesis: the abstraction of hydrogen at C-10 of 5-HpETE.

Adenosine triphosphate

5-LO catalysis is stimulated by several nucleotides with ATP as the most prominent representative [110], [111], [112], whereas other LOs do not bind nucleotides. It was shown that addition of 0.1-2 mM ATP causes a 2- to 6-fold increase in 5-LO activity, and K_α values of 30-100 µM were determined for ATP binding [113], [114]. Stimulation of 5-LO by ATP does not necessarily depend on divalent cations, but Ca^{2+} as well as Mg^{2+} clearly increase the enzyme activity [115], [116]. Since most of the intracellular ATP is bound to Mg^{2+}, it is most likely that a $MgATP^{2-}$ complex instead of free ATP is responsible for 5-LO activation [89].

ATP binding to 5-LO can be utilized for affinity purification of the protein from different sources [117], [118]. Although an ATP binding site within the 5-LO amino acid sequence has not been determined yet, two tryptophan residues (Trp75 and 201) might be involved in ATP association. Thus, analogs of ATP bind specifically to these moieties, and simultaneous application together with ATP prevents 5-LO stimulation by the latter [119]. However, Trp75 could be mutated without a significant alteration in ATP-affinity. A Trp201Arg mutant, in contrast, was hardly expressed, and a clearly attenuated ATP-column yield as well as a dramatically reduced 5-LO activity was received upon replacement of Trp201 by Ala or Ser. The reason of this phenomenon might be a deranged 5-LO structure.

Notably, ATP does not have to be hydrolyzed to exert its stimulatory action on 5-LO, and energy consumption or (auto-)phosphorylation do not occur [112]. Eventually, ATP seems to act on 5-LO by stabilizing its protein structure.

Lipid hydroperoxides

Oxidation of the ferrous iron by LOOHs is essential for 5-LO activation. 5-HpETE, 12-HpETE and 13-HpODE are able to stimulate crude 5-LO in homogenates from PMNL, whereas certain hydroperoxides such as tert-butyl hydroperoxide or cumene hydroperoxide show no efficacy [53], [120]. In addition, 5-LO activity is increased under conditions that elevate lipid peroxidation, and the lag phase of 5-LO that is observed in cell-free assays after addition of AA is shortened upon addition of LOOHs [121].

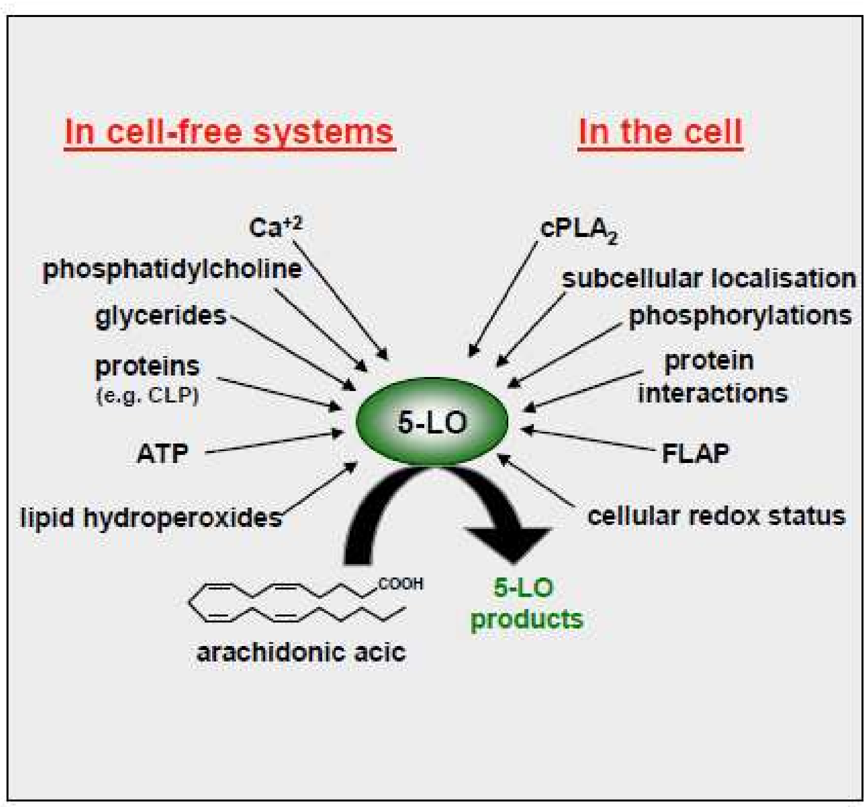


Fig. 7. **Factors that influence 5-LO activity in cell-free systems and in intact cells.** *Abbreviations:* ATP, adenosine triphosphate; CLP, coactosin-like protein; $cPLA_2$, cytosolic phospholipase A_2; FLAP, 5-LO activating protein. (from Werz, O. et al. Expert Opin Ther Patents 15, 2005)

2.3.1.6. Regulation of 5-LO activity in the cell

5-LO activity in the cell is subject to a variety of control mechanisms, and the amount of free AA [122] as well as its accessibility to 5-LO [123] influence the extent of product formation (**Fig. 7**). Hence, cellular 5-LO catalysis depends on the release of AA by $cPLA_2$, localization of 5-LO inside the cell, phosphorylation events, interaction with FLAP and/or other cellular proteins and on the redox tone within the cell. It was shown that most of the stimuli that activate 5-LO (e.g. N-formyl-methionyl-leucyl-phenylalanine (fMLP), platelet activating factor (PAF), opsonized zymosan, LTB_4, C5a, IL-8 and ionophores) also possess a stimulatory potential on $cPLA_2$ [124]. Actually, similarities in the structure (C2 domain) and also the regulation of both enzymes (activation by Ca^{2+} and by phosphorylation) have been demonstrated [125], [28], [55], and both 5-LO and $cPLA_2$ bind to the nuclear membrane after activation of leukocytes.

Subcellular localization and mobility of 5-LO

A variety of studies described 5-LO as a soluble enzyme in resting cells. In particular, the protein was found to be cytosolic in neutrophils, eosinophils and peritoneal macrophages, whereas it was located in the nucleosol associated with chromatin in alveolar macrophages, Langerhans cells or rat basophilic leukaemia cells [124]. Activation of the cell by an appropriate stimulus leads to a translocation of 5-LO (and $cPLA_2$) to the nuclear membrane, a process that is mediated by several factors like Ca^{2+}, phosphorylations and interaction with CLP (**Fig. 8**). Subsequently, transfer of AA liberated from membrane phospholipids by $cPLA_2$ is facilitated by FLAP, and LT formation is initiated. In contrast, addition of exogenous AA does not require 5-LO translocation but occurs in the cytosol. Moreover, transcellular mechanisms allow a supply with AA from neighboring cells [126].

The position of 5-LO in unstimulated cells might be important for redistribution after stimulation. Thus, cytosolic 5-LO seems to translocate to the outer nuclear membrane, whereas association with the inner membrane of the nuclear envelope occurs in cells where 5-LO is inside the nucleus prior to application of a stimulus [123]. In general, cell-types where 5-LO is located in the nuclear matrix (except eosinophils) show a higher tendency to produce LTs than cells with cytosolic 5-LO. Nuclear import of 5-LO is mediated by three nuclear localization sequences (NLS) present in the N-terminal domain and close to the C-terminus [127], [128], [129], [130], [131], [132], [133]. 5-LO was found in the nucleus after treatment of resting cells with glycogen

or cytokines [134], [135] as well as after adherence to surfaces [136], [137]. In addition, recruitment of leukocytes to inflammatory sites seems to be attended by a shift of 5-LO into the nucleus [136], [138]. The regulation of nuclear export is not fully elucidated yet, but it is thought to be mediated by an exportin-l-like mechanism that binds a nuclear export sequence (NES) within 5-LO [139]. It has been proposed already in earlier studies that phosphorylation events are involved in the regulation of nuclear import/export of 5-LO [140], [141], and recently, Flamand et. al. showed that phosphorylation of Ser271 by MK-2/3 retains 5-LO inside the nucleus thus facilitating LT biosynthesis upon 5-LO activation [142]. In contrast, phosphorylation on Ser523 by PKA prevents nuclear import resulting in a suppression of 5-LO catalysis [82], [143].

Regarding 5-LO translocation from a cytosolic location to the nuclear membrane, a gender difference in 5-LO localization and LT biosynthesis was revealed recently. In accordance with previous observations, 5-LO in resting human PMNL from females was found in the cytosol, whereas in male cells, a considerable amount of the enzyme was already present at the perinuclear region prior to activation [144]. It has been suggested in previous findings that 5-LO may be hard to activate if it has already bound to the nuclear membrane [145], [146]. Consequently, LT formation was clearly reduced by androgens in an ERK-dependent manner after stimulation of whole blood or PMNL with fMLP. This effect might explain gender differences in the occurrence of inflammatory diseases involving LTs. Thus, it is well known that asthma or allergic rhinitis are dominant in females [147], [148]. Notably, a gender-specific reduction of atheroma formation in female dual 5-LO and 12/15-LO deficient mice was demonstrated in an animal model of atherosclerosis [149].

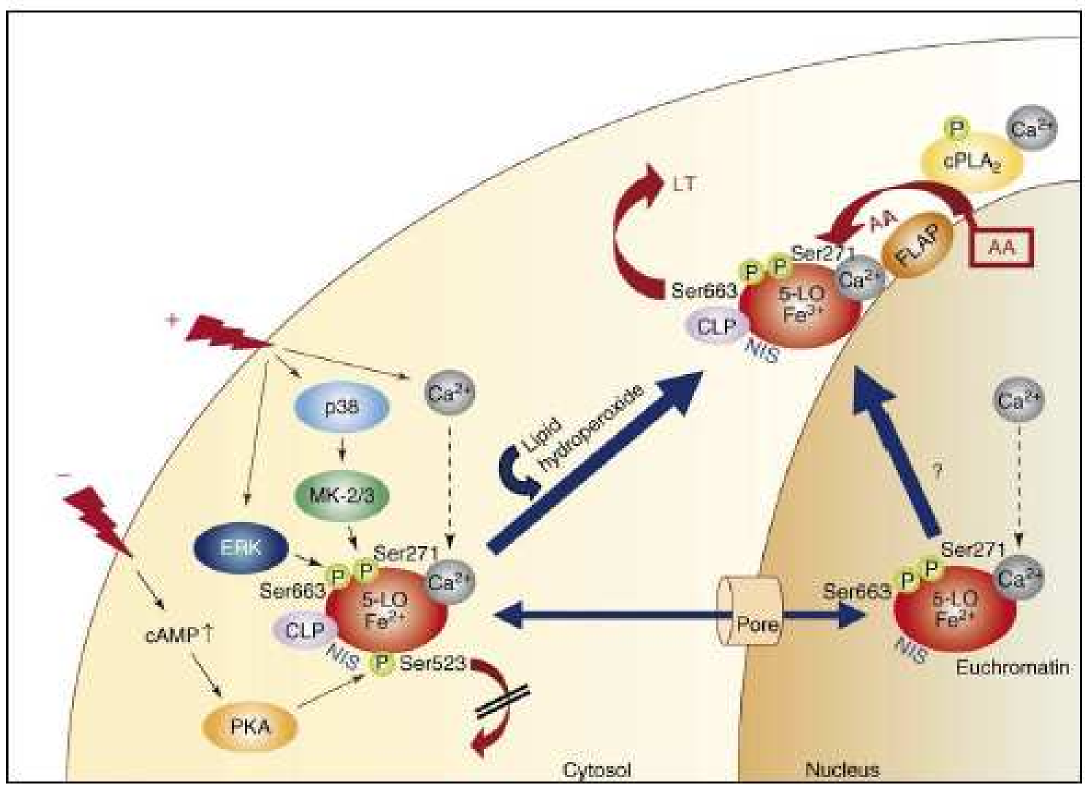


Fig. 8. **5-LO activation in the cell.** Unstimulated 5-LO is found either in the cytosol or in a soluble compartment inside the nucleus. Upon activation, 5-LO translocates to the nuclear envelope where $cPLA_2$ and FLAP are involved in 5-LO product formation. AA from phospholipids is liberated by $cPLA_2$, and membrane-bound FLAP is thought to facilitate the transfer of AA to 5-LO. CLP can translocate together with 5-LO. Nuclear import sequences (NIS) in the 5-LO sequence mediate import into the nucleus. Addition of an appropriate stimulus leads to an increase in Ca^{2+} and/or activation of MAPK. LOOHs oxidize the iron to the ferric form (Fe^{3+}). In 5-LO, Ser271 is phosphorylated by MK-2/3 downstream of p38 MAPK. ERK phosphorylates 5-LO on Ser663. PKA is activated by an increase in cAMP levels and exerts an inhibitory function on 5-LO. (from *Radmark, O. et al. Trends Biochem Sci 32, 332-41, 2007*)

Phosphorylation of 5-LO

LT formation is not only increased upon elevation of intracellular Ca^{2+}, but also after cell stress- or phorbol ester-induced 5-LO phosphorylation by p38 MAPK regulated MK-2/3 (at Ser271) or ERK (at Ser663), respectively [66]. Respective kinase inhibitors abrogated this stimulatory effect on 5-LO product formation [28]. Also, mutation of Ser271 and/or Ser663 to Ala reduced LT synthesis in transfected cells stimulated with AA (that activates MAPK). However, phosphorylation of 5-LO by MK-2/3 or ERK does not increase the product formation in cell-free

assays [80], [81]. Interestingly, it was found that in Chinese hamster ovary (CHO-K1) cells and human embryonic kidney 293 (HEK293) cells, 5-LO is exported from the nucleus after cell stress-induced phosphorylation of Ser271 [150], whereas it was recently demonstrated that in NIH 3T3 cells, phosphorylation at this position results in an inhibition of nuclear export of 5-LO [142]. Thus, instead of directly affecting 5-LO, phosphorylation events at Ser271 or Ser663 rather modulate the interaction of 5-LO with other cellular components.

Increased cyclic adenosine monophosphate (cAMP) levels in response to agents such as adenosine, PGE_2 or β-adrenergic agonists lead to an activation of PKA that phosphorylates 5-LO at Ser523. This implicates an attenuated LT formation in cell free systems as well as in intact cells [82], [151]. It was shown that nuclear import is prevented upon phosphorylation at this site, and the direct inhibitory effect was suggested to be ascribed to allosteric changes close to the active site [143]. Interestingly, exogenous AA is able to prevent the inhibitory effect of cAMP-mediated 5-LO phosphorylation [152]. Also, moderate stimulation of ERK by androgens was shown to attenuate LT formation in PMNL due to an induction of 5-LO translocation to the perinuclear region (as described above) [144].

Influence of the cellular redox tone

The redox tone within cells is a critical parameter for LT synthesis since the active site iron has to be oxidated to induce 5-LO activity (see above). 5-LO product formation is increased under conditions that promote lipid peroxidation (e.g. elevation of ROS by phorbol 12-myristate 13-acetate (PMA), addition of peroxides, inhibition of GPx, or reduced glutathione levels). In contrast, GPx-1 and -4 reduce 5-LO activity by diminishing LOOH levels [124]. Moreover, oxidative stress leads to a phosphorylation of 5-LO mediated by p38 MAPK. Interestingly, the efficacy of certain nonredox-type 5-LO inhibitors (see below) is reduced under these conditions, and it was demonstrated that for potent 5-LO inhibition, these compounds require the presence of GPx activity leading to lowered hydroperoxide levels [153], [154]. It was described that in presence of Ca^{2+} or OAG, LT synthesis occurs also at a low redox tone. Thus, both agents might reduce the amount of LOOHs needed for 5-LO activation, probably because of an increased affinity of the enzyme towards LOOHs [90], [98].

Interaction with 5-LO activating protein and other cellular proteins

In 1990, Miller et al. discovered the membrane bound FLAP as target of a new class of indoles that inhibit cellular LT formation but do not affect the activity of soluble 5-LO [155]. The 18 kDa FLAP belongs to the membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) family, and its crystal structure has been determined in 2007 [156]. The protein crystallizes as a homotrimer that consists of four transmembrane helices linked by two elongated cytosolic loops and one short luminal loop. A direct binding of FLAP and 5-LO has not been shown yet, whereas an *in vitro* association between FLAP and LTC_4S (as well as between 5-LO and LTC_4S) was demonstrated recently [157]. 5-LO and FLAP are often expressed simultaneously [158], [159]. However, upregulation of FLAP but not 5-LO was observed as well [160], [134], and there are some 5-LO negative cells that do express FLAP [161], [162]. Nevertheless, in transfection experiments with a human osteosarcoma cell line, 5-LO alone was not sufficient for LT production although the enzyme was active in homogenates of these cells [163]. Only after cotransfection with FLAP cells were able to synthesize LTs. These findings are supported by the fact that FLAP deficient macrophages from knock-out mice do not produce LTs [164]. Hence, coexistence of FLAP and 5-LO at the nuclear membrane seems to be essential for cellular LT synthesis from membrane phospholipids [50], and it was shown that the efficacy of the FLAP inhibitors MK-886 and licofelone in human PMNL is reduced upon addition of exogenous AA [165]. Taken together, FLAP seems to facilitate the transfer of AA to 5-LO and is therefore required for efficient cellular LT formation.

5-LO contains a Src homology 3 (SH3)-binding motif that mediates association with the SH3-domain of growth factor receptor-bound protein 2 (Grb2), an adaptor protein that is involved in cell signaling by tyrosine kinases [166]. A contribution of the SH3 binding motif in nuclear translocation of 5-LO has been discussed, since a peptide corresponding to this sequence was able to abrogate redistribution of the enzyme to the nuclear envelope after stimulation of PMNL. In addition, apart from being a key enzyme in lipid mediator formation, 5-LO might play a role in tyrosine kinase signaling.

Co-immunoprecipitation experiments in HL-60 cell lysates revealed an interaction of 5-LO with NF-κB [130], preferentially in ionophore stimulated cells. Therefore, it was assumed that 5-LO might be of importance in cellular NF-κB responsiveness.

In a yeast two hybrid screening of a human lung cDNA library with 5-LO as a bait, three proteins that interact with 5-LO were found: i) CLP (as mentioned above); ii) TGFβ receptor-I-associated protein I (TRAP-1); and iii) the ribonuclease Dicer [105], [167]. TGFβ increases 5-LO expression and activity in maturing myeloid cells, and TRAP-1 might functionally interfere with 5-LO. Dicer is involved in the biogenesis of microRNAs (miRNA), and its C-terminus was shown to interact with Trp13, 75 and 102 in the C2-like domain of 5-LO, leading to an enhanced LT synthesis [168]. Besides, 5-LO was able to modify the miRNA precursor processing activity of Dicer so that there might be a relation between miRNA caused regulation of gene expression and chronic inflammation.

2.3.1.7. Receptors and pathophysiological roles of 5-LO products

LTs exert their biological functions via specific GPCRs of the rhodopsin class. These heptahelical receptors can be found on the outer plasma membrane of structural and inflammatory cells. LTB_4 activates BLT_1 and BLT_2 receptors, whereas cys-LTs act on $CysLT_1$ and $CysLT_2$ receptors [169]. Recently, a third cys-LT receptor that binds preferentially LTE_4 has been discovered in animal studies [170]. Finally, 5-Oxo-ETE acts via a highly selective G_i protein-coupled OXE receptor that is expressed in eosinophils, neutrophils and monocytes [171]. 5-Oxo-ETE induces chemotaxis, actin polymerization, Ca^{2+} mobilization, integrin expression, and degranulation [172], [45], and although the pathophysiological role of this metabolite of 5-HpETE is not completely clarified, it seems to be involved in asthma, allergic diseases, cancer and cardiovascular diseases [173].

LTB_4 induces migration of granulocytes and T cells to inflammatory sites and causes activation of these cells. Consequently, granulocytes attach to vessel walls, degranulate and release superoxides as well as the cathelicidin LL-37 [174], [175]. Moreover, LTB_4 increases phagocytosis of neutrophils and macrophages [176] and the secretion of immunoglobulins by lymphocytes [177]. The chemotactic effect of LTB_4 is mediated by the BLT_1 receptor, and it is assumed that atherogenesis, asthma, glomerulonephritis, arthritis and chronic inflammatory bowel diseases require signal transduction via this receptor subtype [178]. The effect of BLT_2 activation, in contrast, is not completely understood, but it might be a kind of substitute receptor for BLT_1 at high LTB_4 levels if the other receptor is desensitized. However, LTB_4 also possesses

anti-inflammatory effects that are exerted through activation of peroxisome proliferator-activated receptor (PPAR)α [179], [180].

Formation of Cys-LTs implicates smooth muscle contraction, plasma extravasation, mucus secretion, vasoconstriction, recruitment of eosinophils and fibrocyte proliferation [174], [181], [182]. It is still unclear if the G_q-protein coupled Cys-LT receptors form homo- and/or hetero-dimers, and how this fact influences their functions [183]. $CysLT_1$ can be found in peripheral blood leukocytes, spleen, lung tissue, smooth muscle cells and macrophages [184], and mediates bronchoconstriction, mucus secretion, and airway edema. The more ubiquitously expressed $CysLT_2$ (present in eosinophils, peripheral blood monocytes, lung macrophages, endothelial cells, etc.) rather contributes to inflammation, tissue fibrosis and endothelial cell activation [51], [185], [186].

Because of their multiple actions, LTs are suggested to be involved in the regulation of the immune response and in the pathogenesis of a variety of inflammatory diseases. The most validated role of these lipid mediators can be found in asthma [187], [188] and allergic rhinitis, but numerous studies in animals and/or humans also indicate a relation between the 5-LO pathway and rheumatoid arthritis, inflammatory bowel disease, psoriasis, shock [189], osteoporosis [190] and certain types of cancer (in particular colon, prostate and pancreatic cancer [191]) (for review, see [28]). Furthermore, LTs seem to essentially contribute to the development of cardiovascular diseases such as atherosclerosis, stroke and myocardial infection (for review, see [192]), and interference with the LT pathway therefore is a promising strategy in the treatment of these widespread diseases [193]. Anti-LT therapy can be pursued either by a suppression of LT synthesis or by the use of LT receptor antagonists. CysLTs antagonists such as montelukast or zafirlukast are successfully used in the treatment of asthma since the late 1990s. However, many LT related diseases may require the concerted suppression of all LTs instead of exclusive interaction with one receptor subtype, and since 5-LO is the key enzyme in LT biosynthesis, intervention with 5-LO product formation proposes a great therapeutic benefit in a variety of inflammatory diseases.

2.3.1.8. Inhibitors of 5-LO product formation

5-LO product formation can be reduced by inhibition of $cPLA_2$, FLAP or 5-LO itself. Blockade of $cPLA_2$ (e.g., by glucocorticoids) leads to a decrease in the availability of AA and thus suppresses the formation of all eicosanoids [7]. Notably, it was shown that neither glucocorticoids nor selective $cPLA_2$ inhibitors exert their anti-inflammatory actions via an inhibition of LT formation [174], [194], [195], [196]. FLAP inhibitors prevent substrate transfer to 5-LO at the nuclear membrane and therefore potently suppress LT formation in intact cells. Notably, most of the compounds developed so far seem to strongly bind to plasma proteins and/or compete with AA and other *cis*-unsaturated fatty acids. However, a very promising candidate is AM103 (**Fig. 9**) that selectively inhibited FLAP in animal models of acute and chronic inflammation [197] and dose dependently reduced blood LTB_4 production in phase II studies [198]. Licofelone (**Fig. 9**), a compound that was originally developed as dual 5-LO and COX inhibitor, has been shown to target FLAP, COX-1 and mPGES-1 but not 5-LO or COX-2 [165], [199] and is currently undergoing phase III of clinical trials for osteoarthritis.

Four different types of direct 5-LO inhibitors can be categorized: redox-active compounds that reduce the active site iron of the enzyme, iron ligand inhibitiors, nonredox-type inhibitors that compete with AA or LOOHs for binding to 5-LO and a novel class of compounds that act in different ways [200].

Fig. 9. Chemical structures of the FLAP inhibitors licofelone and AM103

Redox-active 5-LO inhibitors

Many lipophilic plant-derived substances including flavonoids, coumarins, caffeic acid, nordihydroguaretic acid and several polyphenols possess reducing properties and are therefore able to keep the active site iron of 5-LO in the ferrous state [201]. Besides, several synthetic compounds such as AA-861 (**Fig. 10**) act by uncoupling the catalytic cycle of the enzyme. A major disadvantage of both natural and rationally developed redox-active inhibitors is their poor selectivity for 5-LO. In addition, most of the compounds are ineffective *in vivo* due to oxidation during absorption or a rapid metabolism, and interference with other biological redox systems as well as the production of reactive radical species may cause severe side effects such as the formation of methemoglobin [200].

Fig. 10. Chemical structure of the redox-active 5-LO inhibitor AA-861

Iron-ligand 5-LO inhibitors

Several hydroxamic acids or hydroxyurea derivatives are able to chelate the active site iron of 5-LO. Among those compounds (and in fact among all LT synthesis inhibitors), zileuton (**Fig. 11**) is the only representative that is available on the (US-) market for the treatment of asthma. The drug inhibits 5-LO in stimulated leukocytes with an IC_{50} of 0.5-1 µM and clearly improves airway functions, but nevertheless, no significant benefit was found in allergic rhinitis, rheumatoid arthritis and inflammatory bowel disease [124]. Zileuton was structurally optimized, and its fivefold more potent successor ABT-761 (VIA-2291) (**Fig. 11**) is currently investigated in clinical trials for atherosclerosis and cardiovascular diseases [202].

The hydroxamic acid BWA4C (**Fig. 11**) selectively inhibits 5-LO (IC_{50} = 40 nM, in intact granulocytes) [203], but is rapidly inactivated *in vivo* and forms toxic nitric oxide radicals. However, the compound is widely used as a research tool for *in vitro* experiments.

Zileuton

ABT-761

BWA4C

Fig. 11. Chemical structures of the iron ligand 5-LO inhibitors zileuton, ABT-761, and BWA4C

Nonredox-type 5-LO inhibitors

A class of structurally diverse molecules that do not possess any redox or iron chelating properties has been designated nonredox-type 5-LO inhibitors. These compounds compete with AA or LOOHs for binding to 5-LO, possibly in an allosteric manner [200]. Of interest, the efficacy of some nonredox-type inhibitors (e.g. ZM 230487, **Fig. 12**) is impaired at elevated peroxide levels and/or if 5-LO is activated by phosphorylation [153], [154], both conditions that occur during inflammatory reactions.

However, not all of the nonredox-type 5-LO inhibitors depend on reducing conditions and need 5-LO activation by Ca^{2+}. For example, the more recently developed RBx 7796 [204], [205] and CJ-13,610 [206], [207], [208] (**Fig. 12**) potently suppress 5-LO product formation regardless of the assay conditions, and both of them show *in vivo* effectiveness. Several other nonredox-type inhibitors have been patented during the last years, but none of them has been approved by public authorities so far [200].

CJ-13,610

RBx 7796

ZM230487

Fig. 12. Chemical structures of the nonredox-type 5-LO inhibitors ZM 230487, CJ-13,610 and RBx 7796

Diverse 5-LO inhibitors and dual inhibitors of LT and PG formation

A naturally occurring 5-LO inhibitor is the polyprenylated acylphloroglucinol hyperforin, a lipophilic ingredient of *Hypericum perforatum* (St John's wort, **Fig. 13.**). Hyperforin was shown to inhibit 5-LO (and also COX-1 [209]) in the low micromolar range, and *in vivo* effectiveness was demonstrated in a carrageenan-induced pleurisy model in rats [210]. The compound does not possess any redox or iron-chelating properties, and its characteristics are different from those of nonredox-type 5-LO inhibitors. It was recently shown that the potency of hyperforin is significantly decreased in presence of PC and also when W13, W75 and W102 are mutated to alanine [210]. In addition, the interaction between 5-LO and CLP was diminished, and translocation of the enzyme to the nuclear membrane was inhibited suggesting an interference of hyperforin with the C2-like domain of 5-LO.

Hyperforin

Fig. 13. Chemical structure of the 5-LO inhibitor hyperforin

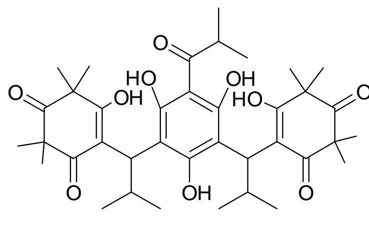
Blockade of both LT and PG synthesis is thought to be more effective in the treatment of inflammatory diseases than inhibition of only one eicosanoid pathway [211], [212]. In addition, dual COX/5-LO inhibitors have been associated with a lower incidence of unwanted side effects on the gastrointestinal tract than classical NSAIDs. This might be ascribed to the concurrent suppression of LTs that were shown to contribute to gastric epithelial injury [51].

It has been demonstrated that the selective COX-2 inhibitor celecoxib as well as the unspecific COX inhibitor sulindac (**Fig. 14**) also influence 5-LO [213], [214], [215], and a variety of compounds that affect both enzymes (some of them show structural similarities with coxibs) have been synthesized during the last years (for review, see [200]). However, COX-2 inhibition may be accompanied by severe cardiovascular side effects [216], and therefore, exclusive suppression of PGE_2 formation in combination with 5-LO inhibition is considered a beneficial alternative to dual COX/5-LO inhibition [18], [217]. Certain lipophilic natural compounds with phenolic structures (e.g. curcumin [218], myrtucommulone from *Myrtus communis* [219] and epigallocatechin-3-gallate from green tea (*Camellia sinensis*) [220] (**Fig. 14**)) have been identified as potent dual inhibitors of 5-LO and mPGES-1 without significant effects on COX enzymes, and anti-inflammatory activities of these compounds have been demonstrated in preclinical or clinical studies.

Celecoxib

Sulindac

Curcumin



Myrtucommulone

Epigallocatechin-3-gallate

Fig. 14. Chemical structures of the dual inhibitors of leukotriene and prostaglandin formation: celecoxib, sulindac, curcumin, myrtucommulone, and epigallocatechin-3-gallate.

2.4. Peroxisome proliferator-activated receptors

PPARs belong to the superfamily of ligand-activated transcription factors and play crucial roles in the regulation of lipid and glucose metabolism but also in inflammatory processes [221]. Transcriptional control occurs after heterodimerization with the retinoid X receptor (RXR) and subsequent binding to a specific DNA sequence element, called peroxisome proliferator response element (PPRE) [222]. Three subtypes of PPAR with different tissue distribution and physiology have been identified. PPARα is expressed in liver, kidney, heart, muscle and cells of the arterial

wall and regulates genes involved in fatty acid uptake and storage, inflammation and glucose metabolism. The receptor is activated endogenously by fatty acids, in particular LTB_4 [180], and pharmacologically by drugs of the fibrate class [223]. PPARβ/δ can be found in endothelial cells, smooth muscle cells and macrophages. Activation of this receptor leads to a reduction of inflammation and apoptosis in the respective cell type [224]. No synthetic PPARβ/δ ligand could enter the market up to now, but strong efforts are made to develop drugs that act on this receptor subtype for the treatment of metabolic syndrome. Glitazones, a class of insulin-sensitizing drugs used against type-2 diabetes target the PPARγ receptor. Activation of this subtype increases fatty acid storage in adipocytes so that plasma free fatty acid levels are reduced, resulting in an improvement of insulin sensitivity in liver and skeletal muscle [225]. Since many patients suffering from type 2 diabetes also exhibit dyslipidemia, the idea came up to develop dual PPARα/γ agonists several years ago. Nevertheless, the so called glitazares (i.e. ragaglitazar, muraglitazar and tesaglitazar) revealed severe side effects like cardiovascular events, anemia or urothelial cancer in clinical studies and had to be withdrawn from phase 3 trials [224]. However, novel dual PPARα/γ agonists with a better safety profile as well as dual PPARγ/δ and PPARα/δ agonists are currently under investigation. In addition, development of tissue and target gene-selective PPAR receptor modulators (SPPARMs) is a promising strategy to overcome adverse effects of full agonists.

The anti-inflammatory effect upon PPAR activation is mediated by a repression of different pathways such as NF-κB, activator protein 1 (AP-1), CCAAT/enhancer binding protein beta (C/EBP beta), signal transducer and activator of transcription 1 (STAT-1) and nuclear factor of activated T-cells (NFAT) signaling [221]. Consequently, the expression of inflammatory cytokines, chemokine receptors and adhesion molecules is reduced, and cell recruitment to inflammatory sites is prevented. Therefore PPAR agonists might also be beneficial in the treatment of chronic inflammatory diseases such as atherosclerosis [226].

2.5. Pirinixic acid and derivatives

4-Chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid (WY-14643), also referred to as pirinixic acid (PA) belongs to the lipid-lowering fibrates that activate PPARα and, to a lower extend, PPARγ. PA was first described in 1974 as an antihypercholesterolemic, but subsequent studies revealed that it rather causes a reduction of triglyceride- than of cholesterol levels, and it was demonstrated in 1990 that its target is PPARα [227], [228], [229]. Despite its high potency in reducing serum lipid levels, PA could never enter the market as a hypolipidemic drug, since a hepatocarcinogenic effect was obvious in rodent animal studies [230]. However, although this effect turned out to be related to receptor activation and was observed for a variety of PPARα agonists in mice and rats, a promotion of liver tumors in humans by these compounds has not been demonstrated so far [231], [232], [233].

Besides acting as a PPARα agonist, PA was shown to upregulate the expression of the TRB3 gene in lymphocytes, resulting in a delay of the G2 phase in the cell cycle and thus a blockade of cell proliferation [234]. Moreover, induction of plasminogen activator inhibitor 1 (PAI-1) gene expression in HepG2 cells was reported [235]. This effect was synergistic with insulin and independent of PPARα, but associated with an activation of MAPKs. While interference of PA with genomic pathways has been demonstrated in numerous studies, little is known about immediate effects of this substance (e.g. a reduction of the activity of human aldose reductase [236] and its ability to uncouple mitochondrial oxidative phosphorylation in isolated mitochondria [237]).

PA is unsubstituted in α-position of the carboxylic group, whereas other fibrates possess a di-methyl group at this site. The group of Prof. Dr. M. Schubert-Zsilavecz (University of Frankfurt) synthesized a series of α-substituted PA derivatives with enlarged hydrophobic aromatic extension (**Fig. 15**) that act as dual PPARα/γ agonists with low micromolar activities [238], [239]. Additional structural variations led to derivatives of 2-(bis-(phenethoxy)-pyrimidine-2-ylthio)-hexananoic acid that influence only PPARγ [240], whereas a series of highly active PPARα agonists was developed based on the structure of 2-(phenylthio)hexanoic acid [241].

Besides acting as PPAR agonists, these novel compounds turned out to potently inhibit LT biosynthesis in PMNL with IC_{50} values in the low micromolar range although PA itself was ineffective in this respect [242], [243], [244].

Pirinixic acid (PA)

PA derivatives

2-(phenylthio)hexanoic acid derivatives

Fig. 15. Chemical structures of PA, PA derivatives and 2-(phenylthio)hexanoic acid. X: S or C; R1: alkyl chain or aryl residue, R2: hydrophobic aromatic residue; R3: Cl, phenylalkyloxy or alkyloxy residue

3. Aim of the study

LTs are potent bioactive lipids that are involved in a variety of pathological conditions such as inflammation, allergy, cardiovascular diseases, cancer and osteoporosis [51], [191]. Since 5-LO catalyzes the first step in LT biosynthesis, it is a promising target for the pharmacological treatment of disorders related to an excess of these lipid mediators [66].

By introducing alkyl chains or aryl groups in α-position of the carboxylic residue of the PPAR agonist PA and by enlarging the hydrophobic part of the molecule, the group of Prof. Dr. M. Schubert-Zsilavecz (University of Frankfurt) synthesized a variety of compounds that do not only possess an increased activity on PPARs but also potently inhibit 5-LO product formation in human PMNL. In addition, a series of highly active PPAR agonists that also suppress 5-LO product formation was developed based on the structure of 2-(phenylthio)hexanoic acid. In the course of this work, structural features that are important for the inhibitory effect on LT biosynthesis were identified, and structure-activity relationships (SARs) of the derivatives were determined.

For the successful development of drugs that are effective in 5-LO related diseases, the complex regulation of 5-LO has to be taken into account, and distinguished experimental settings should be applied for *in vitro* investigations. Thus, the enzymatic activity of 5-LO in cell-free assays is stimulated by Ca^{2+}, PC, glycerides and CLP that all act via the C2-like domain, but also by LOOHs and ATP. All these factors may influence the inhibitory potency of a potential drug candidate. In intact cells, interaction with FLAP or $cPLA_2$, but also interference with 5-LO translocation and the activation pathway of 5-LO have to be considered. Therefore, a detailed biological characterization of 5-LO inhibition was performed with selected compounds out of four different subcategories of PA derivatives or 2-(phenylthio)hexanoic acid derivatives, respectively. Their pharmacological profile was examined regarding characteristics of redoxactive-, iron ligand- or nonredox-type 5-LO inhibitors as well as qualities of C2-like domain-interacting substances. Thus, radical scavenging properties of the compounds, selectivity towards other LOs as well as reversibility of the inhibitory effect were investigated, and dependence on the substrate concentration was determined. In addition, the influence of the cell stimulus and different 5-LO stimulatory factors was addressed, and interference with 5-LO translocation to the nuclear membrane was evaluated. Finally, the effect on 5-LO product

formation in human whole blood was analyzed, and *in vivo* activity of selected derivatives was studied in cooperation with the group of Prof. Dr. L. Sautebin (University of Naples, Italy).

Pharmacological interference with both PGs and LTs is thought to be more effective than inhibition of one single pathway, and in terms of gastrointestinal and cardiovascular side effects, dual 5-LO/mPGES-1 inhibitors seem to be superior over dual 5-LO/COX inhibitors. To exclude interference with COX enzymes, several PA derivatives and 2-(phenylthio)hexanoic acid derivatives were assessed in cell-free assays with isolated ovine COX-1 and human recombinant COX-2 [240]. Parallel to this work, A. Koeberle et al. and M. Hieke et al. investigated inhibition of cell-free mPGES-1 by a variety of compounds, and most of them turned out to affect both 5-LO and mPGES-1 [243].

Interestingly, previous findings in our group revealed quinolinic α-alkyl substituted PA derivatives as potent inhibitors of typical neutrophil functions such as ROS formation, HLE secretion and intracellular Ca^{2+} mobilization [245]. Since most of these functions are elicited after activation of p38 MAPK and ERKs, interference of the compounds with these kinases was investigated in this work.

Finally, it was interesting to know if PA derivatives and 2-(phenylthio)hexanoic acid derivatives also interfere with other targets in neutrophils. Therefore, selected compounds were either immobilized directly to an insoluble resin via their carboxylic function, or a linker group was synthetically introduced (in the group of Prof. Dr. M. Schubert-Zsilavecz) and the compound was subsequently bound to an insoluble polymeric matrix. These constructs were incubated with cell lysates from neutrophils, and proteins that bound specifically to the bait molecule could be precipitated, separated by SDS-PAGE or 2-D-DIGE, and identified by MALDI mass spectrometry. Since fishing experiments can only reveal a physical interaction of two binding partners but do not provide information about the functional consequences, the physiological relevance of this interaction should be clarified in appropriate assay systems.

4. Materials and methods

4.1. Materials

10% non-immune goat serum	Invitrogen (Darmstadt, Germany)
11β-PGE_2	Cayman Chemical (Ann Arbor, MI, USA)
13(S)-HpODE	Cayman Chemical (Ann Arbor, MI, USA)
5-LO antibody (affinity-purified anti-5-LO antiserum, 1551, AK7)	generous gift by Prof. Dr. O. Rådmark, Karolinska Institute (Stockholm, Sweden)
AA	Sigma-Aldrich (Deisenhofen, Germany)
Acetonitrile	Sigma-Aldrich (Deisenhofen, Germany)
Acrylamide solution 30% (37.5:1)	AppliChem (Darmstadt, Germany)
Ada	Merck (Darmstadt, Germany)
AG® 501-X8 Resin	Bio-Rad Laboratories, Inc. (Hercules, CA, USA)
Agar-Agar	Merck (Darmstadt, Germany)
Alexa Fluor 488 goat anti-rabbit IgG	Invitrogen (Darmstadt, Germany)
ALP-conjugated IgGs	Sigma-Aldrich (Deisenhofen, Germany)
Amersham™ ECL Western Blotting detection reagent	GE Healthcare (Freiburg, Germany)
Amersham Hyperfilm™ ECL	GE Healthcare (Freiburg, Germany)
APS	AppliChem (Darmstadt, Germany)
Ampholyte Bio-Lyte3/10®	Bio-Rad Laboratories, Inc. (Hercules, CA, USA)

ATP	Roche Diagnostics (Mannheim, Germany)
ATP-Agarose	Sigma-Aldrich (Deisenhofen, Germany)
BCIP	AppliChem (Darmstadt, Germany)
BioRad DC Protein Assay Kit	Bio-Rad Laboratories, Inc. (Hercules, CA, USA)
β-mercaptoethanol	Carl Roth (Karlsruhe, Germany)
Bromophenol blue	Merck (Darmstadt, Germany)
BWA4C	generous gift by Dr. L. G. Garland (Wellcome Research Laboratories, London, UK)
Ca^{2+}-ionophore A23187	Sigma-Aldrich (Deisenhofen, Germany)
Celecoxib	WITEGA Laboratories (Berlin, Germany)
Chaps	AppliChem (Darmstadt, Germany)
Coomassie brilliant blue G250	AppliChem (Darmstadt, Germany)
COX-1 (ovine)	Cayman Chemical (Ann Arbor, MI, USA)
COX-2 (human recombinant)	Cayman Chemical (Ann Arbor, MI, USA)
CyDye DIGE Fluor, minimal labeling kit	GE Healthcare (Freiburg, Germany)
Dextrane	Sigma-Aldrich Inc. (St. Luis, MO, USA)
DMEM/High glucose (4.5 g/l) medium	PAA (Coelbe, Germany)
DMF 99,8% extra dry	Acros Organics (Geel, Belgium)
DMEM with high glucose (4.5 g/l)	PAA (Pasching, Austria)
DMSO	Carl Roth (Karlsruhe, Germany)
DPPH	Sigma-Aldrich (Deisenhofen, Germany)

DTT	Sigma-Aldrich (Deisenhofen, Germany)
Dulbeccos buffer substance	Serva Electrophoresis (Heidelberg, Germany)
ECL Plex goat-anti-mouse IgG-Cy3	GE Healthcare (Munich, Germany)
ECL Plex goat-anti-rabbit IgG-Cy5	GE Healthcare (Munich, Germany)
EDTA	Sigma-Aldrich (Deisenhofen, Germany)
FAD	Sigma-Aldrich (Deisenhofen, Germany)
Fatty acid-free BSA	Sigma-Aldrich (Deisenhofen, Germany)
FCS	Sigma-Aldrich (St. Luis, MO, USA)
fMLP	Sigma-Aldrich (Deisenhofen, Germany)
GSH, reduced	Sigma-Aldrich (Deisenhofen, Germany)
GSH-Sepharose™4B	GE Healthcare (Freiburg, Germany)
HBS-P buffer	GE Healthcare (Freiburg, Germany)
HEPES solution	PAA (Pasching, Austria)
Hybond™-C Extra nitrocellulose membranes	GE Healthcare (Freiburg, Germany)
Hyperforin dicyclohexylammonium	Generous gift by Dr. Willmar Schwabe GmbH&Co (Karlsruhe, Germany)
IL-1 β	ReproTech (Hamburg, Germany)
Indomethacin	Sigma-Aldrich (Deisenhofen, Germany)
IPG Strips (ReadyStrip™) 17 cm 3-10NL	Bio-Rad Laboratories, Inc. (Hercules, CA, USA)

IPTG	Applichem (Darmstadt, Germany)
Lamin B antibody	Abcam (Cambridge, UK)
Leupeptin	AppliChem (Darmstadt, Germany)
LPS	Sigma-Aldrich (Deisenhofen, Germany)
LSM 1077 Lymphocyte Separation Medium	PAA (Coelbe, Germany)
LTB_4 EIA kit	Assay Designs (Ann Arbor, MI, USA)
LTB_4 RIA kit	Assay Designs (Ann Arbor, MI, USA)
Lysozyme from chicken egg white	Sigma-Aldrich (Deisenhofen, Germany)
MBP-5-LO	kindly provided by Prof. Dr. D. Steinhilber (University of Frankfurt, Germany)
Methanol	Merck (Darmstadt, Germany)
Mineral oil	Bio-Rad Laboratories, Inc. (Hercules, CA, USA)
MK-886	BioTrend Chemicals (Cologne, Germany)
Mowiol	Merck (Darmstadt, Germany)
MTT	Sigma-Aldrich (Deisenhofen, Germany)
NBT	Roche Diagnostics (Mannheim, Germany)
N,N'-Diisopropylcarbodiimide	Acros Organics (Geel, Belgium)
Nonidet® P40	AppliChem (Darmstadt, Germany)
NQO2 human recombinant	Sigma-Aldrich (Deisenhofen, Germany)
NQO2 polyclonal antibody (mouse)	Abcam (Cambridge, UK)

OAG	Sigma-Aldrich (Deisenhofen, Germany)
p38 MAPK antibody	Abcam (Cambridge, UK)
PAF C-16	Cayman Chemical (Ann Arbor, MI, USA)
PC	Sigma-Aldrich (Deisenhofen, Germany)
Penicillin / Streptomycin solution	PAA (Coelbe, Germany)
peqGold Protein Marker IV	peqLab Biotechnology (Erlangen, Germany)
Peroxidase-conjugated IgGs	Sigma-Aldrich (Deisenhofen, Germany)
PGB_1	Sigma-Aldrich (Deisenhofen, Germany)
PGH_2	Larodan (Malmö, Sweden)
phospho-ERK1/2 (Thr202/Tyr204) antibody	Cell Signalling (Boston, MA, USA)
phospho-p38 MAPK (Thr180/Tyr182) antibody	Cell Signalling (Boston, MA, USA)
PMSF	Sigma-Aldrich (Deisenhofen, Germany)
Ponceau S red	Sigma-Aldrich (St. Luis, MO, USA)
Poly-L-lysine (MW 150,000-300,000)	Sigma-Aldrich (Deisenhofen, Germany)
Protease Inhibitor Cocktail P8340	Sigma-Aldrich (St. Luis, MO, USA)
PS	Sigma-Aldrich (Deisenhofen, Germany)
SDS	Carl Roth (Karlsruhe, Germany)
Sensor Chip CM 5	GE Healthcare (Freiburg, Germany)
Silver nitrate	Sigma-Aldrich (St. Luis, MO, USA)

STI	Sigma-Aldrich (Deisenhofen, Germany)
Streptomycin	PAA (Pasching, Austria)
TFA	AppliChem (Darmstadt, Germany)
THF	Carl Roth (Karlsruhe, Germany)
Thiourea	AppliChem (Darmstadt, Germany)
Toyopearl AF-Amino 650M	Tosho Bioscience (Stuttgart, Germany)
Toyopearl AF-Epoxy 650M	Tosho Bioscience (Stuttgart, Germany)
Triton X-100	Carl Roth (Karlsruhe, Germany)
Trypan Blue	AppliChem (Darmstadt, Germany)
Trypsin/EDTA solution	PAA (Pasching, Austria)
Tween 20	Carl Roth (Karlsruhe, Germany)
Urea	AppliChem (Darmstadt, Germany)
WY-14643 (Pirinixic Acid)	Sigma-Aldrich (Deisenhofen, Germany)
ZM230487	generous gifts by Dr. R. M. McMillan (Zeneca Pharmaceuticals, Macclesfield, UK)
λ-carrageenan type IV isolated from Gigartina aciculaire and Gigartina pistillata	Sigma-Aldrich (Milan, Italy)

All other chemicals were purchased in analytical grade from Sigma-Aldrich (Deisenhofen, Germany) unless stated otherwise.

Pirinixic acid derivatives and 2-(phenylthio)hexanoic acid derivatives

PA derivatives and 2-(phenylthio)hexanoic acid derivatives were synthesized by M. Dittrich, M. Hieke, L. Popescu, Y. Syha, T. Thieme and H. Zettl in the group of Prof. Dr. M. Schubert-Zsilavecz (University of Frankfurt, Germany) as described [238], [239], [243], [240], [241], [246]. Stocks of the compounds were prepared at concentrations ≤ 30 mM in DMSO and kept at -20 °C. Freezing-thawing cycles were kept to a minimum.

4.2. Animals[a]

Wistar Han rats (190-200 g, Harlan, Milan, Italy) and CD-1 mice (26-30 g) 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).

[a]In cooperation with Prof. Dr. L. Sautebin (University of Naples, Italy)

4.3. Methods

4.3.1. Isolation of human PMNL

Human PMNL were freshly isolated from leukocyte concentrates obtained at the Blood Center of the University Hospital Tuebingen (Germany). In brief, venous blood was taken from healthy adult donors, and leukocyte concentrates were prepared by centrifugation at 4,000×g for 20 min at 20 °C. PMNL were immediately isolated by dextran sedimentation, centrifugation on LSM 1077 Lymphocyte Separation Medium and hypotonic lysis of erythrocytes as described previously [247]. Cells were finally resuspended in phosphate-buffered saline pH 7.4 (PBS) containing 1 mg/ml glucose and 1 mM $CaCl_2$ (PGC buffer).

4.3.2. Determination of product formation of 5-LO, 15-LO and 12-LO in intact cells

For assays of intact cells stimulated with Ca^{2+}-ionophore A23187 or NaCl, 5 × 10^6 freshly isolated PMNL were resuspended in 1 ml PGC buffer. After preincubation with test compounds for 15 min at 37 °C (and addition of further compounds as specified), 5-LO product formation was started by addition of 2.5 µM Ca^{2+}-ionophore A23187 and exogenous AA as indicated.

Alternatively, 0.3 M NaCl was supplemented 3 min before addition of AA (without A23187). After 10 min at 37 °C, the reaction was stopped with 1 ml MeOH and 30 µl HCl (1 N), 200 ng PGB_1 as well as 500 µl of PBS were added. Formed 5-LO metabolites were extracted and analyzed by HPLC as described [248]. 5-LO product formation is expressed as ng of 5-LO products per 10^6 cells, which includes LTB_4 and its all-trans isomers 5(S),12(S)-DiHETE and 5-H(p)ETE. Cysteinyl LTC_4, -D_4 and -E_4 were not detected, and oxidation products of LTB_4 were not determined. 15-H(p)ETE was analyzed as product of 15-LO and 12-H(p)ETE as product of 12-LO. To assess 5-LO product formation in cells stimulated with fMLP, 2×10^7 freshly isolated PMNL were resuspended in 1 ml PBS/glucose. Cells were primed with 1 µg LPS for 10 min at 37 °C and 0.3 U Ada was added. After another 10 min, cells were treated with test compounds for 10 min, and 1 µM fMLP was added to start 5-LO product formation. The reaction was stopped on ice after 5 min, and cells were centrifuged (800×g, 10 min). The amount of released LTB_4 was determined by EIA according to the manufacturer's protocol.

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

E.coli BL21 cells were transformed with pT3-5LO plasmid, and recombinant 5-LO was expressed at 37 °C as described [249]. For isolation of 5-LO protein, *E.coli* were lysed by incubation in 50 mM triethanolamine (TEA)/HCl pH 8.0, 5 mM EDTA, soybean trypsin inhibitor (STI, 60 µg/ml), 1 mM phenylmethylsulfonyl fluoride (PMSF) and lysozyme (500 µg/ml), homogenized by sonication (3 × 15 s) and centrifuged at 40,000×g for 20 min. To partially purify 5-LO, the 40,000×g supernatant (S40) was applied to an ATP-agarose column. The column was eluted as described previously [250]. Purified 5-LO or S40 were immediately used for 5-LO activity assays.

4.3.4. Determination of 5-LO product formation in cell-free systems

When whole cell homogenates were assayed, 5×10^6 freshly isolated PMNL were resuspended in 1 ml of PBS containing 1 mM EDTA and sonicated (3 × 10 s). Cell disruption was verified by trypan blue staining. Cell homogenates were either used immediately for 5-LO activity assays, or centrifuged at 100,000×g for 1 h at 4 °C, and 5-LO products in the resulting supernatant (S100) were assessed. For determination of the activity of recombinant 5-LO, either the S40 supernatants from *E.coli* lysates (corresponding to 4 ml *E.coli* culture) or 0.5 µg partially purified 5-LO were

diluted with PBS/EDTA. Aliquots from PMNL homogenates or recombinant 5-LO were supplemented with 1 mM ATP and pre-incubated with test compounds and additional reagents (e.g., DTT, OAG or PLs) as indicated. After 5-10 min at 4 °C, samples were pre-warmed for 30 s at 37 °C, and 2 mM $CaCl_2$ (if indicated) and the appropriate amounts of AA with or without 13(S)-HpODE or 30 µM AA alone were added to start 5-LO product formation. The reaction was stopped after 10 min at 37 °C by addition of 1 ml ice-cold methanol, and the formed metabolites were analyzed by HPLC as described for intact cells.

4.3.5. Determination of 5-LO activity in human whole blood

Freshly withdrawn venous blood was collected by the Blood Center, University Hospital (Tübingen, Germany) in heparinized tubes (S-Monovettes Sarstedt, 13.1628, 16 I.E. Heparin/mL blood) by venipuncture from fasted (12 h) male and female volunteers, with consent. The blood was divided in 2 mL aliquots in glass vials, pre-incubated 10 min at 37° C with test compounds and then stimulated with Ca^{2+}-ionophore A23187 (30 µM) for 10 min. The reaction was stopped on ice, and samples were centrifuged at 600×g, 10 min, 4° C. Aliquots of plasma (500 µL of supernatant) were mixed with 2 ml methanol, and PGB_1 (200 ng) was added as internal standard. Samples were put at -20° C for 2 h and then centrifuged again (600×g, 15 min, 4° C). Supernatants were collected and diluted with 2.5 ml PBS plus 75 µL HCl 1N. Formed 5-LO metabolites were extracted and analyzed by HPLC as described for PMNL.

4.3.6. Analysis of subcellular redistribution of 5-LO

PMNL (3×10^7) in 1 ml PGC buffer were pre-incubated with test compounds for 15 min at 37 °C, stimulated with 2.5 µM Ca^{2+}-ionophore A23187 for 5 min or primed with LPS (1 µg/ml) and Ada (0.3 U/ml) followed by stimulation with fMLP (1 µM) for 5 min at 37 °C and chilled on ice. Alternatively, cells were incubated with test compounds in presence of 0.3 U Ada for 20 min at 37 °C and immediately chilled on ice. Subcellular localization of 5-LO was determined either by mild detergent (0.1% nonidet P-40) lysis [251] or by sonication of the cells followed by preparation of soluble (S100) and membrane (P100) fractions by 100,000×g centrifugation [252], respectively. 5-LO in these fractions was analyzed by SDS-PAGE and Western Blot using 5-LO antibody.

4.3.7. Indirect Immunofluorescence Microscopy

Human PMNL (1.5×10^6, freshly isolated from buffy coats of female donors), were resuspended in 1 mL ice-cold PGC buffer. Cells were pretreated with test compounds for 15 min at 37 °C and then centrifuged at 30×g for 1 minute onto poly-L-lysine (MW 150,000-300,000)-coated glass coverslips in the wells of a 12-well plate. Cells were activated by addition of 2.5 µM Ca^{2+}-ionophore A23187, for 3 min at 37° C. Cells were fixed in methanol (-20° C, 30 min) and permeabilized with 0.1% Tween 20 in Dulbecco's PBS (RT, 10 min), followed by 3 washing steps with PBS. Samples were blocked with 10% non-immune goat serum for 10 min at RT and then washed again twice with PBS. 5-LO staining was performed by incubating the coverslips with 5-LO antibody for 1 h at RT. The coverslips were washed ten times with PBS, incubated with Alexa Fluor 488 goat anti-rabbit IgG (diluted 1:1,500 in PBS) for 10 min at RT in the dark and washed ten times with PBS. The DNA was stained with 0.1 µg/ml diamidino-2-phenylindole (DAPI) in PBS for 3 min at RT in the dark. The coverslips were washed ten times and mounted on glass slides with Mowiol containing 2.5% n-propyl gallate. Fluorescence was visualized with a Zeiss Axiovert 200M microscope using a 100X oilimmersion objective.

4.3.8. Glutathione-S-transferase (GST) pull-down assay

Recombinant human CLP was expressed as GST fusion protein using the plasmid pGEX-5X-1-CLP and purified as described [106], [107]. 20 µg of purified GST-CLP linked to GSH-Sepharose 4B beads (20 µl of 50% slurry) was incubated with partially purified 5-LO protein (5 µg) in the presence of 50 µg bovine serum albumin (BSA) and the indicated amount of test compounds in 200 µl buffer A (2 mM Tris-HCl pH 8.0, 0.2 mM ATP, 0.2 mM $CaCl_2$, 2 mM $MgCl_2$, 50 mM KCl, 0.5 mM β-mercaptoethanol). After 30 min gentle rotation at RT, beads were washed five times with buffer A (without BSA). Bound proteins were eluted from the beads with 200 µl of elution buffer (10 mM GSH in 50 mM Tris-HCl, pH 8.0), for 60–90 min at RT. Beads were sedimented, and 5-LO in the supernatant was analyzed by SDS-PAGE and Western Blot using 5-LO antibody.

4.3.9. Surface plasmon resonance (SPR) experiments

Recombinant human 5-LO expressed as MBP fusion protein was kindly provided by Prof. Dr. D. Steinhilber (University of Frankfurt, Germany) [253]. SPR experiments were carried out on a

BIAcore® X device (GE Healthcare Freiburg, Germany). MBP-5-LO (75 µg/ml) in 10 mM Na-acetate pH 4.5 was coupled to a carboxymethylated dextran surface (CM-5 chip) using standard amine coupling chemistry according to the manufacturer's directions. There are two flow cells on each chip: MBP-5-LO (5.25 µg) was immobilized to flow cell 2 corresponding to 6400 resonance units (RU), whereas flow cell 1 was loaded with the equivalent amount of substance of MPB alone (corresponding to 2327 RU) as reference. Equilibration of the baseline was completed by passing a continuous flow of HBS-P buffer (10 mM HEPES, 150 mM NaCl, 0.01% surfactant P20 and 1% DMSO, pH 7.4) through the chip for 2 h. During the SPR experiments, the stock solution of test compounds (dissolved in DMSO) was diluted into assay buffer. All measurements were performed at 25 °C and a flow rate of 30 µl/min.

After recording association, the liquid phase was replaced by assay buffer, and the dissociation was monitored. The binding profiles were obtained after subtracting the response signal of the MBP reference cell 1, and sensograms were processed using automatic correction for nonspecific bulk refractive index effects using BIAEVALUATION Version 3.1 software (GE Healthcare).

4.3.10. Determination of antioxidant activity

The indicated amounts of test compounds were incubated with 50 µM diphenylpicrylhydrazyl (DPPH) in ethanol for 30 min at RT in a 96 well plate, shaking. Reduction of the absorbance at 520 nm was measured with a multiwell scanning spectrophotometer (Victor plate reader, PerkinElmer) [254].

4.3.11. Carrageenan-induced pleurisy in rats

Experiments were conducted by F. Dehm in the laboratory of Prof. Dr. L. Sautebin (University of Naples, Italy). Male Wistar Han rats (200-220 g, Harlan, Milan, Italy) were anesthetized with enflurane 4% mixed with O_2, 0.5 l/min, 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 skin incision was closed with a suture, 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. The chest was carefully opened, and the pleural cavity was rinsed with 2 ml saline solution containing heparin (5 U/ml). The exudate and washing solution were removed by aspiration, and the total volume was measured. Any

exudate that was contaminated with blood was discarded. The amount of exudate was calculated by subtracting the volume injected (2 ml) from the total volume recovered. Leukocytes in the exudate were resuspended in PBS and counted with an optical light microscope in a Burker's chamber after vital trypan blue staining. In the treated group of animals, test compound **22** and **37** (1.5 mg/kg, each) was given i.p. 30 min before carrageenan. Indomethacin (5 mg/kg) was used as reference compound, and vehicle-treated group of rats received DMSO (4% (v/v), i.p.) 30 min before carrageenan. The amount of PGE_2, 6-keto-$PGF_{1\alpha}$ and LTB_4 in the supernatant of centrifuged exudate (800×g for 10 min) was assayed by radioimmunoassay (RIA) and enzyme immunoassay (EIA), respectively, according to manufacturer's protocol.

4.3.12. Platelet activating factor (PAF)-induced shock in mice

Experiments were conducted by A. Rossi in the laboratory of Prof. Dr. L. Sautebin (University of Naples, Italy). PAF was injected intravenously into female mice at a dose of 200 µg/kg. Test compound **39** (1.5, 5, 10 mg/kg) or vehicle control (saline solution containing 2% DMSO) were administered i.p. 30 min prior to PAF. The animals were monitored closely for 1-2 h, and the number of surviving animals was assessed.

4.3.13. Cell culture - A549 cells

A549 (human lung epithelial carcinoma cell line) cells were obtained from Prof. Dr. O. Rådmark (Karolinska Institute, Stockholm, Sweden). 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). After 3 days, confluent cells were detached using 1× trypsin/ethylenediaminetetraacetate (EDTA) solution and reseeded at 2×10^6 cells in 20 ml medium.

4.3.14. Cell viability[b]

Cell viability of A549 cells was measured using the colorimetric 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay [255] in a 96-well format. Briefly, A549 cells (4×10^4 cells/100 µl medium) were plated into a 96-well microplate and incubated at 37 °C and 5% CO_2 for 16 h. Then, 30 µM of test compound or solvent (DMSO) was added, and samples were incubated for another 5 h. MTT (20 µl, 5 mg/ml) was added, and the incubations were continued for 4 h. Alternatively, A549 cells were stimulated with IL-1β (2 ng/ ml) and

incubated with test compounds or vehicle for 24 h at 37 °C. MTT (20 µl, 5 mg/ml) was added, and the incubations were continued for 1 h. The formazan product was solubilized with sodium dodecylsulfate (SDS) (10%, (w/v) in 20 mM HCl), and the absorbance of each sample was measured at 595 nm relative to that of vehicle (DMSO)-treated control cells using a multiwall scanning spectrophotometer (Victor[3] plate reader, PerkinElmer, Rodgau-Juegesheim, Germany). Neither PA nor any of the tested derivatives (**22**, **30**, **37**, **39**, **25**, **136** or **86** at 10 µM final concentration, each) significantly reduced cell viability versus DMSO as vehicle (data not shown), excluding possible acute cytotoxic effects of the compounds in the cellular assays using A549 cells.

[b] performed by A. Koeberle in the lab of Prof. Dr. O. Werz (University Tuebingen, Germany)

4.3.15. Induction of mPGES-1 in A549 cells and isolation of microsomes

Preparation of A549 cells was performed as described [17]. In brief, cells (2×10^6 cells in 20 ml DMEM/High glucose (4.5 g/l) medium containing FCS (2%, v/v) were incubated for 16 h at 37 °C and 5% CO_2. Subsequently, the culture medium was replaced by fresh medium, IL-1β (1 ng/ml) was added, and cells were incubated for another 72 h. Thereafter, cells were detached with trypsin/EDTA, washed with PBS and frozen in liquid nitrogen. Ice-cold homogenization buffer (0.1 M potassium phosphate buffer pH 7.4, 1 mM PMSF, 60 µg/ml STI, 1 µg/ml leupeptin, 2.5 mM GSH and 250 mM sucrose) was added, and after 15 min, cells were resuspended and sonicated on ice (3 × 20 s). 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 Coomassie protein assay.

4.3.16. Determination of PGE_2 synthase activity in microsomes of A549 cells

Microsomal membranes of A549 cells were diluted in potassium phosphate buffer (0.1 M, pH 7.4) containing 2.5 mM GSH (100 µl total volume), and test compounds or vehicle (DMSO) were added. After 15 min, PGE_2 formation was initiated by addition of PGH_2 (20 µM, final concentration). After 1 min at 4 °C, the reaction was terminated with 100 µl of stop solution (40 mM $FeCl_2$, 80 mM citric acid, and 10 µM 11β-PGE_2), PGE_2 was separated by solid phase extraction on reversed phase (RP)-C18 material using acetonitrile (200 µl) as eluent and analyzed

by RP-HPLC (30% acetonitrile aqueous + 0.007% TFA (v/v), Nova-Pak® C18 column, 5 × 100 mm, 4 µm particle size, flow rate 1 ml/min) with UV detection at 195 nm. 11β-PGE_2 was used as internal standard to quantify PGE_2 product formation by integration of the area under the peaks.

4.3.17. Activity assays of isolated COX-1 and COX-2

Inhibition of the activities of isolated ovine COX-1 and human recombinant COX-2 was performed as described [256], [257]. Though not of human origin, ovine COX-1 is generally used for inhibitor studies when examining the effectiveness of compounds on the activity of isolated COX-1 enzyme [257]. Briefly, purified COX-1 (ovine, 50 units) or COX-2 (human recombinant, 20 units) were diluted in 1 ml reaction mixture containing 100 mM Tris-HCl pH 8, 5 mM GSH, 5 µM hemoglobin, 100 µM EDTA at 4 °C and pre-incubated with the test compounds for 5 min. Samples were pre-warmed for 60 s at 37 °C, and AA (5 µM for COX-1, 2 µM for COX-2) was added. After 5 min at 37 °C, the reaction was terminated by addition of 1 ml methanol, and the COX product 12-HHT was extracted and analyzed by HPLC as described [209], [258].

4.3.18. Determination of MAPK activation

Human PMNL (10^7/100 µl PGC buffer) were preincubated with test compounds (or 0.3% DMSO as vehicle) for 15 min. Then, fMLP (1 µM) was added for 1.5 min at 37 °C, the reaction was stopped by addition of 100 µl of 2× SDS loading buffer (20 mM Tris-HCl, pH 8, 2 mM EDTA, 5% (m/v) SDS, 10% (v/v) β-mercaptoethanol). Samples were vortexed and boiled for 6 min at 95° C. Total cell lysates were analyzed for phosphorylated ERK1/2, phosphorylated p38 MAPK and p38 MAPK by SDS-PAGE and Western Blot.

4.3.19. Preparation of whole cell lysates from PMNL

Human PMNL were lysed in 1 ml lysis buffer (50 mM HEPES pH 7.4, 200 mM NaCl, 1 mM EDTA, 1% Triton X-100, 50 µl P8340 protease inhibitor cocktail) per g drained cell pellet. After sonification on ice (5 × 10 s) and centrifugation (100,000×g, 1 h, 4 °C), the supernatants were immediately used for protein fishing experiments.

4.3.20. Immobilisation of PA derivatives to amino-functionalised matrix

500 µl Toyopearl AF-Amino-650M dry beads were prewashed with Milli-Q water, 50% DMF and DMF (5 ml each). Compounds **24**, **143, 144** or PA, respectively, were solubilized in DMF (100 µmol/500 µl) and mixed with washed beads. After addition of 65 µl N,N′-Diisopropylcarbodiimide (DIC), the pH was adjusted to 4.5, and samples were rotated at RT for 48 h. Afterwards, the resin was washed with DMF, 50% DMF and Milli-Q water (10 ml each), and after resuspension in 1 ml Milli-Q water, remaining free amino groups were blocked with 42.5 µl acetic acid (10%) and 100 µl DIC at RT over night. Then, beads were washed three times with 1 ml DMF and three times with 2 ml acetate buffer (0.1 M sodium acetate, 0.1 M acetic acid, pH 4, 0.5 M NaCl) and Tris buffer (0.1 M Tris-HCl pH 8, 0.5 M NaCl), alternatively, to remove ionic bound ligand. The beads were finally washed with 1 ml Milli-Q water and 1 ml 20% (v/v) ethanol and stored at 4 °C in 20% (v/v) ethanol until use.

4.3.21. Deprotection of the ester groups of compounds 143 and 144

100 µmol Toyopearl AF-Amino-650M coupled with compound **143** or **144**, respectively, was mixed with 300 µmol LiOH in 2.68 ml THF (75%) and rotated for 24 h at RT to cleave the ethylester of the compounds. Beads were washed three times with 1 ml THF and three times with 2 ml acetate buffer (0.1 M sodium acetate, 0.1 M acetic acid, pH 4, 0.5 M NaCl) and Tris buffer (0.1 M Tris-HCl pH 8, 0.5 M NaCl), alternatively. The beads were finally washed with 1 ml Milli-Q water and 1 ml 20% (v/v) ethanol, and stored at 4 °C in 20% (v/v) ethanol until use.

4.3.22. Immobilisation of 2-(phenylthio)hexanoic acid derivatives to epoxy-functionalised matrix

75 mg Toyopearl AF-Epoxy-650M dry beads were prewashed with Milli-Q water, 50% DMF and DMF (5 ml each). Compound **145** or **146**, respectively, were solubilized in DMF (40 µmol/400 µl) and mixed with washed beads. After addition of 100 µl NaOH (1 M), samples were rotated at RT for 72 h. The resin was washed with DMF, 50% DMF and Milli-Q water (3 ml each), and after resuspension in 1 ml Milli-Q water, remaining free epoxy groups were blocked with 60 µl ethanolamine plus 10 µl NaOH (1 M) at RT over night. Afterwards, beads were washed and stored as described in **4.3.20.**

4.3.23. Protein fishing assays

50 µl compound-coupled beads were mixed with 500 µl Milli-Q water, washed three times by centrifugation (7,000×g, 7 min, 4 °C) and finally resuspended in 500 µl Milli-Q water. The beads were incubated with PMNL lysate (corresponding to the indicated amount of cells) in 700 µl binding buffer (50 mM HEPES pH 7.4, 200 mM NaCl, 1 mM EDTA) with the indicated amount of Triton X-100 for 12 h at 4 °C, rotating. After three washing steps with 500 µl binding buffer plus Triton X-100 1%), fished proteins were eluted by addition of 50 µl SDS-PAGE loading buffer (20 mM Tris pH 8.0, 2 mM EDTA, 0.5% (w/v) SDS, 10% (v/v) β-mercaptoethanol) followed by 5 min boiling at 96 °C and separation from beads by centrifugation (11,000×g, 10 min, 4 °C). Alternatively, proteins were eluted by incubation with 50 µl 4 M urea (purified from carbamoylated contaminations and salt impurities by ion exchange resin) for 30 min at 4 °C, centrifugation of the beads and subsequent boiling (as described above). Fished proteins were separated either by one dimensional gel electrophoresis (SDS-PAGE) or by two-dimensional gel electrophoresis (2D-PAGE).

4.3.24. SDS-PAGE

Aliqots of protein sample preparations (10 µl) were mixed with the same volume of 2× SDS loading buffer, boiled for 6 min at 95 °C and sonified if necessary. Samples were mixed with 5 µl of glycerol/0.1% bromophenol blue (1:1, v/v), and SDS-PAGE was performed on 8%, 10% or 12% polyacrylamide (PAA) gels using a Mini Trans-Blot Cell system or a Protean II xi Cell system (both from Bio-Rad, Hercules, CA, USA). Molecular weight of the proteins was estimated by comparison with the prestained broad range molecular weight marker peqGOLD IV.

4.3.25. Silver staining

Silver staining was performed by fixing the gels immediately after SDS-PAGE in fixing solution (50% (v/v) ethanol, 40% (v/v) Milli-Q water, 10% (v/v) acetic acid) for 30 min. All steps from then on were performed on ice. Gels were washed for 30 min with Milli-Q water and sensitized by addition of sensitizing solution (30% (v/v) ethanol, 8 mM $Na_2S_2O_3$, 500 mM $C_2H_3NaO_2$, 60% (v/v) Milli-Q water) for 30 min. Again, gels were washed (three times for 5 min with Milli-Q water), and staining solution (30 mM $AgNO_3$) was added for 30 min. After another washing step with Milli-Q water (5 min), the developing solution (235 mM Na_2CO_3, 0.0185% formaldehyde,

0.0025% $Na_2S_2O_3$ in Milli-Q water) was added until an optimal intensity of the staining was reached. Then, gels were quenched in stopping solution (5% acetic acid) and washed with Milli-Q water for 5 min. Pictures of the stained gels were taken with a CCD camera system (CabUVis, Hitachi HV-C20M 3CCD, Sarstedt AG Co., Nümbrecht, Germany).

4.3.26. Western Blot

Gels were electroblotted to nitrocellulose membranes (tank blotting method) after SDS-PAGE, and correct loading of the gels was confirmed by Ponceau S staining. Membranes were blocked with 5% (w/v) BSA blocking buffer (50 mM Tris-HCl, pH 7.4 and 100 mM NaCl (Tris-buffered saline (TBS)) plus 0.1% Tween 20 (TBS-Tween)) for 1 h at RT, washed and incubated with primary antibodies (diluted 1:1,000 in TBS-Tween containing 5% BSA) over night at 4 °C. After several washing steps with TBS-Tween, membranes were incubated with 1:1,000 dilution of alkaline phosphatase (ALP)-conjugated IgGs for 3 h at room temperature. After washing with TBS-Tween and TBS, proteins were visualized with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) in detection buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). Alternatively, development was performed via fluorescence detection. Therefore, membranes were incubated with ECL Plex CyDye-conjugated secondary antibodies (Cy5-conjugated anti-rabbit or Cy3-conjugated anti-mouse) diluted 1:2,500 in TBS-Tween for 1 h at RT. After washing, the membranes were dried at 37° C for 1 h in the dark and then scanned in the Cy3 (Excitation Filter: 540 nm; Emission Filter: 595 nm) or in the Cy5 (Excitation Filter: 635 nm; Emission Filter: 680 nm) channels, respectively, using an Ettan™ DIGE imaging system (GE-Healthcare). In some experiments, membranes were developed via chemoluminescence. In this case, peroxidase-conjugated secondary antibodies were applied in a 1:10,000 dilution for 1 h at RT. After washing (2 × 5 min, 1 × 15 min and 2 × 5 min with TBST buffer), proteins were visualized by addition of Amersham™ ECL Western Blotting detection reagent. Exposed chemoluminescence films (Amersham Hyperfilm™ ECL) were developed using an X-Ray table-top processor (CP 1000, AGFA Healthcare N.V., Mortsel, Belgium).

4.3.27. CyDye™ DIGE Fluor labeling for differential gel electrophoresis (DIGE)

The protein concentration of urea eluted fishing-samples was determined using an urea-compatible protein determination kit (BioRad DC Assay-Kit). CyDye™ DIGE Fluors (Cy3 and Cy5) were diluted 1:2.5 with 3 µl water-free DMF (<0.005 ppm water), and pH was adjusted to

pH 8.5-9.5. This CyDye^{TM} DIGE Fluor working solution (1 µl for 10 µg protein) was added to the samples (Cy5 to the sample with proteins of interest and Cy3 to the negative control) and incubated for 30 min at 4 °C. The reaction was stopped by addition of 1 µl 10 mM lysine. CyDye^{TM} DIGE Fluor labeled samples were stored at -80 °C until isoelectric focusing (IEF).

4.3.28. 2D-PAGE/DIGE

The first dimension of the protein separation was performed by isoelectric focusing (IEF) on immobilized pH gradient (IPG) strips (17 cm, non linear pH gradient from pH 3-10). Cy5- and Cy3-labeled samples were mixed and diluted with PCT buffer (8 M urea, 2 M thiourea, 4% (w/v) CHAPS, ampholyte pH 3-10 0.7% (v/v), 65 mM DTT, 10 µg/ml bromophenol blue, 50 µl P8340 protease inhibitor cocktail per g protein) to a total volume of 300 µl. The solution was pipetted in a 17 cm focusing tray, and an IPG strip was placed on the sample and covered with mineral oil. Rehydration of the strip was performed at 50 V (12 h, 10 °C) in an IEF cell (BioRad Protein® IEF cell, Herecules, CA). Afterwards, the electrode wicks were moistened with 5 µl Milli-Q water and placed on the electrodes. IEF was performed over 17 h with a programmed voltage slope from 0 to 10,000 V. Focused strips were stored at -80 °C until performance of SDS-PAGE.

For development of the second dimension, thawed IPG-strips were incubated two times with equilibration buffer I (6 M urea, 4% (w/v) SDS, 0.05 M Tris pH 8.8, 30% (v/v) glycerol, 1% (w/v) DTT) for 7 min and two times with equilibration buffer II (6 M urea, 4% (w/v) SDS, 0.05 M Tris pH 8.8, 30% (v/v) glycerol, 4% (w/v) iodoacetamide) for 7 min followed by 1×10 s shaking in SDS-PAGE running buffer (200 mM glycine, 25 mM Tris, 3 mM SDS). The IPG strip was placed on top of a 18×20 cm PAA gel (8%, 10% or 12%) and ingrained in an agarose gel (0.5% (w/v) agarose, 0.001% bromophenol blue in SDS-PAGE running buffer (25 mM Tris, 190 mM glycine, 3,5 mM SDS)). Gel electrophoresis was performed using a Protean II xi Cell System. Molecular weight of the proteins was estimated by comparison with a prestained broad range molecular weight marker peqGOLD IV that was applied on a filter paper and placed besides the IPG strip into the agarose gel. Gels were scanned in the Cy3 (Excitation Filter: 540 nm; Emission Filter: 595 nm) and in the Cy5 (Excitation Filter: 635 nm; Emission Filter: 680 nm) channels by an Ettan^{TM} DIGE imaging system (GE-Healthcare).

4.3.29. Mass spectrometry (MS)

MS analysis of fished proteins was performed in cooperation with the Proteom Center Tuebingen via in-gel digestion and nanoflow liquid chromatography tandem MS (nano-LC-ESI-MS/MS) on a QSTAR Pulsar hybrid qTOF mass spectrometer (AB-MDS Sciex) as described previously [259]. Bioinformatic analysis of the identified peptides was subsequently carried out with Matrix Science Mascot.

4.3.30. NRH:quinone oxidoreductase 2 (NQO2) pull-down assay

Toyopearl AF-Amino-650M coupled with compound **24** (50 µl) was incubated with 0.5 µg human recombinant NQO2 in 500 µl binding buffer (50 mM HEPES pH 7.4, 200 mM NaCl, 1 mM EDTA) plus 1% Triton X-100 in the presence of 0.1 µg/ml BSA for 12 h at 4 °C under rotation. Beads were washed three times with binding buffer + 1% Triton X-100, boiled with 2× SDS loading buffer for 5 min at 96 °C, and Western Blot was performed using anti-NQO2 polyclonal antibody .

4.3.31. NQO2 activity assay

Human recombinant NQO2 (10 ng) was pretreated with test compounds, resveratrol (10 µM each) or vehicle in 200 µl assay buffer (25 mM Tris-HCl pH 7.5, 0.01% Tween 20, 0.18 mg/ml BSA, 1 µM flavin adenine dinucleotide (FAD) and 0.5 mM 1-(3-sulfonatopropyl)-3-carbamoyl-1,4-dihydropyrimidine (SCHP)) for 5 min at RT. The enzyme reaction was started with menadione (100 µM) plus 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.134 mg/ml), and the absorbance (at 590 nm) was measured at the indicated times.

4.3.32. Statistics

Statistical evaluation of the data was performed by one-way ANOVA followed by Bonferroni or Tukey-Kramer *post-hoc* test for multiple comparisons, respectively. A p value < 0.05 (*) was considered significant.

5. Results

5.1. Investigation of the 5-LO inhibitory effect of PA derivatives and 2-(phenylthio)hexanoic acid derivatives

5.1.1. SARs of PA derivatives

Inhibition of 5-LO product formation was first investigated in a cell-based assay with intact PMNL. Cells were preincubated with test compounds or vehicle for 15 min at 37 °C and then stimulated with 2.5 µM ionophore A23187 in presence of 20 µM AA (to circumvent substrate supply by $cPLA_2$). As shown in **Table 1**, PA (10 µM) itself did not suppress 5-LO product formation whereas esterification of the carboxylic acid (**1**) led to a weak inhibitory effect. Replacement of the *o*-dimethylaniline moiety of PA by 6-aminoquinoline (**2**, **3**) was beneficial but only for the esterified form (**3**) (see also [242]). Exchange of nitrogen by oxygen as bridging atom (**4, 5**) almost abolished the influence on 5-LO product formation, but insertion of an additional methylene bridge restored the inhibitory potency (**6**). Interestingly, substitution of *o*-dimethylaniline by 2,3-dihydro-indene (**7**, **8**) led to IC_{50} values between 3 and 4 µM for the ester as well as for the free acid. It was previously shown that introduction of alkyl residues in α-position of the carboxylic group of PA increased the potency at PPARα/γ [239]. A similar effect was evident for 5-LO inhibition. Thus, the α-methyl substituted compound **10** was able to reduce 5-LO product synthesis much more efficiently than compound **1** but again the acidic form was inactive. The α-methyl substituted ester carrying a 6-aminoquinoline moiety instead of the *o*-dimethylaniline (**12**) was equipotent with its unsubstituted form, whereas insertion of *n*-butyl- or *n*-hexyl-substituents increased the potency leading to IC_{50} values of 1.1 µM (**16**) and 0.6 µM (**25**), respectively. Of interest, with *n*-alkyl chains longer than methyl (**11**), the free acids (**15**, **24**) became active as well. This effect was also observed for dimethyl (**13**), *n*-hexyl (**22**) and *n*-octyl (**44**) (but not *n*-butyl, **14**) substituted PA (see also [243]).

In addition, further exchanges of the *o*-dimethylaniline residue of α-alkyl substituted PA were performed. Introduction of 3-pyridylethylamine (**17**, **18**) was detrimental compared to 6-aminoquinoline (**15**, **16**) at the *n*-butyl-substituted compound. Exchange of the *o*-dimethylaniline of the *n*-hexyl substituted **22** or its ester **23** by phenethylamine (**19**) or phenoxyethylamine (**20**, **21**) failed to increase the potency on 5-LO as well. In contrast, esterified compounds with

quinoline residues (**27** and **29**) were highly active (IC_{50} = 1.7 and 1.4 µM, respectively), regardless of the exact linkage to the pyrimidine ring. The corresponding acids (**26**, **28**) were significantly less potent whereas the opposite effect was observed when quinoline was replaced by other aromatic residues. The 3,5-bis(2,2,2-trifluoroethoxy)-aniline-substituted derivative **30**, for example, inhibited cellular 5-LO product formation by 50% at 1 µM while its esterified analog **31** was essentially inactive. Along these lines, insertion of 4-(4-chlorophenyl)thiazol-2-ylamine (**32, 33**) or 4-(phenylamino)phenylamine (**34, 35**) yielded 5-LO inhibitors that act in nanomolar concentrations, but only in their acidic form (**32**, **34**). In contrast, an IC_{50} of 4.4 µM was found for compound **36** possessing a 2-(trifluormethyl)-1H-benzo[d]imidazol-5-ylamino residue. Replacement of the *o*-dimethylaniline by biphenylamine moieties led to a series of very potent derivatives. The free acids of compounds with a biphenyl-4-yl-methane amine (**37**), a biphenyl-4-yl-amine (**39**) or a biphenyl-3-yl-amine (**43**) residue but not their corresponding esters (**38**, **40**) showed IC_{50} values below 1 µM, and substitutions at the biphenylic residue were tolerated as well. Thus, a cyano group in para position of the biphenyl (**41**) even increased the potency to inhibit 5-LO with an IC_{50} of 0.4 µM. For this compound, the influence of the stereo center in α-position of the carboxylic group was determined. Both enantiomeric forms (separated by preparative enantioselective HPLC) inhibited 5-LO product synthesis, but the *R*-form (**41a**) was slightly superior over the *S*-configurated form (**41b**) (IC_{50} = 0.15 versus 0.7 µM). 5-methoxy-2-methyl-substitution at the biphenyl residue (**42**) yielded an IC_{50} of 0.5 µM.

Table 1: Inhibition of 5-LO product formation by α-alkyl substituted PA derivatives

compound	R1	R2	R3	R4	5-LO product formation IC_{50} [µM] *or remaining activity (± S.E.) at 10 µM* PMNL	purif. 5-LO
PA (WY-14,643)		-Cl	-H	-H	n.i.[a,b]	n.i.[b]
1		-Cl	-H	$-C_2H_5$	*77.7 % (±0.8)*	n.d.
2		-Cl	-H	-H	n.i. [b]	n.d.
3		-Cl	-H	$-C_2H_5$	5.6	n.d.
4		-Cl	-H	-H	n.i. [b]	n.d.
5		-Cl	-H	$-C_2H_5$	*77.6 % (±5.8)*[b]	n.d.
6		-Cl	-H	$-C_2H_5$	4.5	n.d.
7		-Cl	-H	-H	3.9 [b]	0.8

compound	R1	R2	R3	R4	5-LO product formation IC_{50} [µM] *or remaining activity (± S.E.) at 10 µM* PMNL	purif. 5-LO
8		-Cl	-H	$-C_2H_5$	3.2 [b]	*81.4 % (±6.0)*
9		-Cl	$-CH_3$	-H	n.i. [b]	n.d.
10		-Cl	$-CH_3$	$-C_2H_5$	*22.8 % (±3.3)* [b]	n.d.
11		-Cl	$-CH_3$	-H	n.i. [b]	n.d.
12		-Cl	$-CH_3$	$-C_2H_5$	*12.0 % (±3.6)* [b]	n.d.
13		-Cl	$-(CH_3)_2$	-H	*83.8 % (±7.4)* [b]	n.i. [b]
14		-Cl		-H	n.i. [b]	n.d.
15		-Cl		-H	10.0 [b]	n.d.
16		-Cl		$-C_2H_5$	1.1 [b]	n.d.

compound	R1	R2	R3	R4	**5-LO product formation** IC_{50} [µM] *or remaining activity (± S.E.) at 10 µM* PMNL	purif. 5-LO
17		-Cl	CH_3	-H	*81.7 % (± 3.7)*	*57.3 % (± 3.5)*
18		-Cl	CH_3	$-C_2H_5$	*51.7 % (± 6.4)*	n.i.
19		-Cl	CH_3	-H	3.0	n.i.
20		-Cl	CH_3	-H	4.5	*53.7 % (± 7.1)*
21		-Cl	CH_3	$-C_2H_5$	7	*55.0 % (± 9.7)*
22		-Cl	CH_3	-H	4.1 [b]	7.0
23		-Cl	CH_3	$-C_2H_5$	7.0	n.d.
24		-Cl	CH_3	-H	*20.1 % (± 1.9)* [b]	*53.0 % (± 5.6)*
25		-Cl	CH_3	$-C_2H_5$	0.6 [b]	*90.6 % (± 4.2)*
26		-Cl	CH_3	-H	5.8	n.d.

compound	R1	R2	R3	R4	5-LO product formation IC_{50} [µM] *or remaining activity (± S.E.) at 10 µM* PMNL	purif. 5-LO
27		-Cl	CH_3	$-C_2H_5$	1.7 [b]	n.d.
28		-Cl	CH_3	-H	7.0	*69.1 % (±0.7)*
29		-Cl	CH_3	$-C_2H_5$	1.4 [b]	n.i.
30	CF_3 CF_3	-Cl	CH_3	-H	1.0 [b]	1.9
31	CF_3 CF_3	-Cl	CH_3	$-C_2H_5$	n.i. [b]	n.d.
32	Cl	-Cl	CH_3	-H	0.6	2.7
33	Cl	-Cl	CH_3	$-C_2H_5$	*92.3 % (±6.0)*	*88.6 % (±14.7)*
34		-Cl	CH_3	-H	0.26	1.6
35		-Cl	CH_3	$-C_2H_5$	*52.4 % (±12.4)*	*51.2 % (±15.7)*

compound	R1	R2	R3	R4	5-LO product formation IC_{50} [µM] *or remaining activity (± S.E.) at 10 µM* PMNL	purif. 5-LO
36		-Cl		-H	4.4	*65.9 % (± 11.8)*
37		-Cl		-H	0.9	2.1
38		-Cl		$-C_2H_5$	*80.2 % (±9.9)*	*79.0 % (± 10.1)*
39		-Cl		-H	0.7	1.5
40		-Cl		$-C_2H_5$	*91.2 % (±4.3)*	*79.9 % (± 12.0)*
41		-Cl		-H	0.4	2
41a		-Cl		-H	0.15	1.0
41b		-Cl		-H	0.7	1.8
42		-Cl		-H	0.5	1.5
43		-Cl		-H	0.6	2.0

compound	R1	R2	R3	R4	5-LO product formation IC50 [µM] *or remaining activity (± S.E.) at 10 µM* PMNL	purif. 5-LO
44	H_3C, CH_3, $\overset{H}{N}$ (2,3-dimethylphenylamino)	-Cl	CH_3 (n-octyl chain)	-H	1.1	*93.2 % (±9.8)*

n.i. = no inhibition; n.d. = not determined; S.E. = standard error

[b]data was performed in the lab of Prof. Dr. D. Steinhilber (University of Frankfurt, Germany)

Another synthetic approach was the introduction of phenyl moieties in α-position instead of *n*-alkyl groups (**Table 2**). While the esters of α-phenyl-substituted PA (**46**) and the corresponding 6-aminoquinoline derivative (**48**) potently suppressed 5-LO product formation, the respective acids (**45** and **47**) were ineffective. Compounds with a phenethylamine residue also showed a better inhibition when the carboxylic group was esterified (**50** versus **49**). The α-phenyl-substituted 3,5-bis(2,2,2-trifluoroethoxy)-aniline derivative (**51**) was slightly more potent than the corresponding *n*-hexyl-substituted form (**30**). Remarkably, the corresponding ester (**52**) showed a similar efficacy in this case. Insertion of a biphenyl-4-methane amine moiety at the pyrimidine core together with α-phenyl-substitution (**52**) led to an IC_{50} of 1.8 µM versus 0.9 µM for the compound with the hexyl chain in α-position (**37**). Furthermore, 4′-cyano-biphenyl-4-yl-oxy (**55**) as well as 3′-cyanobiphenyl-4-yl-oxy (**57**) residues were tolerated with only a slight loss of the potency (IC_{50} between 2 and 3 µM). It has to be emphasized that for biphenylic compounds with α-phenyl residues, the esters were less potent than the free acids as it was found for derivatives with α-alkyl substituents.

Table 2: Inhibition of 5-LO product formation by α-phenyl substituted PA derivatives

compound	R1	R2	R3	R4	5-LO product formation IC_{50} [µM] or *remaining activity (± S.E.) at 10 µM* PMNL	purif. 5-LO
45		-Cl		-H	n.i. [b]	n.d.
46		-Cl		$-C_2H_5$	1.8 [b]	n.i.
47		-Cl		-H	*69.1 % (±8.9)* [b]	n.d.
48		-Cl		$-C_2H_5$	1.2 [b]	*93.0 % (± 10.4)*
49		-Cl		-H	*64.5 % (± 16.4)*	*77.1 % (± 5.6)*
50		-Cl		$-C_2H_5$	6.0	*83.8 % (± 12.3)*
51		-Cl		-H	0.7	6.5
52		-Cl		$-C_2H_5$	0.5	*76.1 % (± 5.5)*

compound	R1	R2	R3	R4	**5-LO product formation** IC$_{50}$ [µM] or *remaining activity (± S.E.) at 10 µM* PMNL	purif. 5-LO
53		-Cl		-H	1.8	*7.3 % (± 1.4)*
54		-Cl		-C_2H_5	*56.1 % (± 7.8)*	*77.3 % (± 9.3)*
55		-Cl		-H	2.9	9.5
56		-Cl		-C_2H_5	*88.2 % (± 8.5)*	*88.8 % (± 6.7)*
57		-Cl		-H	2.1	*64.8 % (± 16.2)*
58		-Cl		-C_2H_5	*89.5 % (± 19.6)*	n.i.

n.i. = no inhibition; n.d. = not determined; S.E. = standard error

[b]data was performed in the lab of Prof. Dr. D. Steinhilber (University of Frankfurt, Germany)

In the next step, a series of PA derivatives with α-naphthyl residues was synthesized and subjected to SAR evaluations (**Table 3**, see also [260]). IC_{50} values around 2.5 µM were obtained when the *o*-dimethylaniline moiety of PA was replaced by *n*-butylamine (**59**), isopentylamine (**60**) or phenethylamine (**61**). Prolongation of phenethylamine to phenylpropylamine (**62**) slightly increased the inhibitory effect on 5-LO product formation (IC_{50} = 1.8 µM). α-Naphthyl substituted PA possessed an IC_{50} value of only 5 µM but interestingly, exchange to a 3,4-dimethylaniline-residue (**64**) lowered the IC_{50} to 0.7 µM. Bulkier substituents like 4-isopropylphenylamine (**65**) or 3,5-di-tert-butylphenylamine (**66**) even enhanced the efficacy against 5-LO (IC_{50} = 0.26 µM for both compounds). Upon insertion of chloride in para- or meta-position of the phenylamine residue (**67**, **68**), the IC_{50} was still below 1 µM, whereas fluoride was less tolerated (**69**). Compound **70** possesses a 4-benzamidophenylamine group and still blocks 5-LO with a high efficacy (IC_{50} = 0.6 µM). A biphenyl-4-yl-methane amine at the pyrimidine (**71**) was as active as the corresponding α-hexyl derivative. Deletion of the methylene bridge yielded **72** that was even more potent than the α-hexyl-substituted **39**. With an IC_{50} of 100 nM, this compound represented the most active PA derivative tested so far (equipotent with BWA4C in the cellular 5-LO assay). Repositioning of the biphenyl into biphenyl-3-yl-amine (**73**) as well as bridging of the two rings of the biphenyl (**74**) impaired the potency (IC_{50} = 0.5 µM and 0.24 µM, respectively). The α-naphthyl analog of **41**, namely **75**, with a cyano group in para-position of the biphenyl moiety suppressed 5-LO almost as good as **72** (IC_{50} = 0.17 µM). In addition, several other compounds with different aromatic moieties were tested in this assay. Thus, cyclohexylphenylamine (**76**), phenoxyphenylamine (**77**) and benzylphenylamine (**78**) reduced 5-LO product formation to 50% in concentrations below 0.5 µM. Also compounds with 2,3-dihydro-1H-inden-5-yl-amine (**79**) and naphthalene-2-yl-amine (**80**) did not forfeit their capacity to decrease 5-LO product synthesis. Exchange of naphthylamine to 6-aminoquinoline (**81**) was unfavorable. However, based on the structure of **24**, naphthyl-substitution in α-position of the carboxylic group (**81**) was advantageous compared to hexyl- or phenyl-residues. Finally, an IC_{50} of 0.4 µM was determined for the α-4-phenylnaphthalen-1-yl substituted derivative of PA (**82**).

Table 3: Inhibition of 5-LO product formation by α-naphthyl substituted PA derivatives

compound	R1	R2	R3	R4	5-LO product formation IC50 [µM] or *remaining activity (± S.E.) at 10 µM* PMNL	purif. 5-LO
59	H N	-Cl		-H	2.4	*70.6 % (± 15.2)*
60	H N	-Cl		-H	2.5	*54.8 % (± 8.2)*
61	H N	-Cl		-H	2.5	*44.2 % (± 12.3)*
62	H N	-Cl		-H	1.8	*68.9 % (± 3.5)*
63	CH_3 H_3C H N	-Cl		-H	5.0[b]	n.d.
64	H_3C H_3C H N	-Cl		-H	0.7	8.5
65	H N	-Cl		-H	0.26	5.8

compound	R1	R2	R3	R4	**5-LO product formation** IC$_{50}$ [µM] or *remaining activity (± S.E.) at 10 µM*	
					PMNL	purif. 5-LO
66		-Cl		-H	0.26	1.9
67		-Cl		-H	0.8	8.9
68		-Cl		-H	0.7	5.0
69		-Cl		-H	3.1	*67.3 % (± 2.9)*
70		-Cl		-H	0.6	5.2
71		-Cl		-H	0.7	3.0
72		-Cl		-H	0.1	2.3
73		-Cl		-H	0.5	5.0

compound	R1	R2	R3	R4	5-LO product formation IC_{50} [µM] or *remaining activity (± S.E.) at 10 µM* PMNL	purif. 5-LO
74		-Cl		-H	0.24	1.6
75		-Cl		-H	0.17	2.3
76		-Cl		-H	0.4	2.0
77		-Cl		-H	0.4	3.1
78		-Cl		-H	0.3	2.2
79		-Cl		-H	0.5	4.2
80		-Cl		-H	0.5	2.9
81		-Cl		-H	4.4	*19.0 % (± 2.5)*

compound	R1	R2	R3	R4	5-LO product formation IC_{50} [µM] or *remaining activity (± S.E.) at 10 µM* PMNL	purif. 5-LO
82		-Cl		-H	0.4	2.2

n.i. = no inhibition; n.d. = not determined; S.E. = standard error

[b]data was performed in the lab of Prof. Dr. D. Steinhilber (University of Frankfurt, Germany)

Inhibition of LT synthesis in cellular assays can occur due to direct 5-LO inhibition but also as the result of FLAP or $cPLA_2$ inhibition, interference with MAPKs or Ca^{2+} release or suppressed 5-LO translocation to the nuclear membrane [200]. To investigate if 5-LO is directly affected by PA derivatives, a cell-free assay was performed with human recombinant 5-LO that was partially purified from *E. coli* lysates. Notably, none of the esterified compounds was able to suppress isolated 5-LO effectively. Thus, **21** and **35** were the only active esters with IC_{50} values of approximately 10 µM. The acidic PA derivatives emerged as direct 5-LO inhibitors although they were about 2- to 10-fold less efficient in cell-free assays than in PMNL. For the majority of the free acids, the IC_{50} values at partially purified 5-LO were determined between 1 and 10 µM with **7** as the most active compound (IC_{50} = 0.8 µM).

Taken together, replacement of the *o*-dimethylaniline of PA by bulky lipophilic substituents (especially biphenylic residues) together with introduction of a hexyl chain or a naphthyl moiety in α-position yielded potent 5-LO inhibitors with IC_{50} values in the nanomolar range.

Precedent SARs of PA derivatives included exchanges of the *o*-dimethylaniline moiety and insertion of lipophilic residues in α-position of the carboxylic group of PA. In order to further expand the SARs, derivatives containing two phenethoxy residues at the pyrimidine core (exemplified by 2-(bis-(phenethoxy)-pyrimidine-2-ylthio)-hexananoic acid, **87**) were synthesized and emerged as very potent 5-LO inhibitors [261] (**Table 4**). Therefore, a novel series of compounds with broad variations of i) the two residues at the pyrimidine and ii) of the length of the alkylic chain in α-position was set up based on **87**. Due to the poor efficacy of the ester of **87** (i.e. **88**), the carboxylic acid group was kept for all the other compounds. The *R*-form of **87a** possesses only a slightly lower IC_{50} value (0.8 µM versus 1.3 µM for the *S*-form **87b**) so that the absolute configuration seems not to be essential for the inhibitory effect.

Since the lipophilic chain in α-position of the carboxylic acid was a crucial factor for the potency of PA derivatives (as shown in chapter in **5.1.1.**), its importance for bis-phenethoxy-substituted compounds was evaluated as well. As expected, the unsubstituted compound **83** and the ethyl-substituted compound **85** (IC_{50} = 7.6 and 2.8 µM, respectively) were inferior to compound **87**, whereas introduction of α-hexyl (**86**) and α-phenyl (**84**) did not impair 5-LO inhibition (IC_{50} = 0.4 and 0.8 µM, respectively).

In the next step, the two phenethoxy moieties were subjected to shortening or elongation of their ethylene-spacers. While phenylmethoxy residues (**89**) were detrimental, insertion of propylene (**90**) or butylene (**91**) spacers retained efficacy of the derivatives. α-Butyl-substitution was kept constant for further variations at the lipophilic backbone because of a better solubility and a lower molecular weight compared to hexyl or phenyl-substituted compounds.

Further optimization of **87** could be reached by insertion of different substituents in *para*-position of both phenethoxy moieties. Thus, electron pushing (methyl (**92**), methoxy (**93**)) as well as electron drawing (trifluormethyl (**94**), trifluormethoxy (**95**), nitro (**96**), cyano (**97**)) groups were beneficial with IC_{50} values between 0.3 (**93**) and 0.7 µM (**97**). Additionally, replacement of both phenyl groups by bioisosteric thiophenes (**98**) resulted in a comparable potency (IC_{50} = 0.6 µM).

Moreover, the necessity for aromatic moieties at the lipophilic backbone was investigated. Introduction of isopropyl (**99**) or cyclopropyl (**100**) residues instead of the phenyl groups of **87** lead to a slightly decreased effect on 5-LO (IC_{50} = 1.2 µM for both of them). Enlargement of the

aliphatic ring size to cyclopentyl (**101**) or cycloheptyl (**103**) was detrimental whereas cyclohexyl (**102**) possesses almost the same potency as **87**.

Table 4: Inhibition of 5-LO product formation by PA derivatives with two phenylalkyloxy or alkyloxy residues:

comp.	R1	R2	R3	R4	5-LO product formation IC50 [µM] or *remaining activity (± S.E.) at 10 µM* PMNL	purif. 5-LO
83	O	O	-H	-H	7.6	*57.0 % (± 9.6)*
84	O	O		-H	0.8	4.9
85	O	O	CH_3	-H	2.8	9.0
86	O	O	CH_3	-H	0.4	2.8
87	O	O	CH_3	-H	0.6	4.7
87a	O	O	CH_3	-H	0.8	8.0

comp.	R1	R2	R3	R4	5-LO product formation IC$_{50}$ [µM] or *remaining activity (± S.E.) at 10 µM* PMNL	purif. 5-LO
87b	O	O	CH_3	-H	1.3	7.5
88	O	O	CH_3	$-C_2H_5$	*80.1 % (± 9.1)*	*91.7 % (± 12.4)*
89	O	O	CH_3	-H	1.8	5.0
90	O	O	CH_3	-H	0.9	2.7
91	O	O	CH_3	-H	0.7	2.2
92	H_3C, O	H_3C, O	CH_3	-H	0.5	3.1
93	H_3C, O, O	H_3C, O, O	CH_3	-H	0.3	7.0
94	F_3C, O	F_3C, O	CH_3	-H	0.5	2.2
95	F_3C, O, O	F_3C, O, O	CH_3	-H	0.5	*56.4 % (± 13.3)*
96	O_2N, O	O_2N, O	CH_3	-H	0.5	2.8

comp.	R1	R2	R3	R4	5-LO product formation IC$_{50}$ [µM] or *remaining activity (± S.E.) at 10 µM* PMNL	purif. 5-LO
97	NC, O	NC, O	CH_3	-H	0.7	6.6
98	S, O	S, O	CH_3	-H	0.6	9.3
99	H_3C, CH_3, O	H_3C, CH_3, O	CH_3	-H	1.2	8.8
100	O	O	CH_3	-H	1.2	9.9
101	O	O	CH_3	-H	4.0	6.8
102	O	O	CH_3	-H	0.8	9.0
103	O	O	CH_3	-H	2.4	*53.8 % (± 11.0)*

S.E. = standard error

Next, SARs regarding the importance of the two proton-accepting nitrogens within the pyrimidine ring was investigated (**Table 5**). Therefore, pyrimidine was first replaced by a benzene moiety (**104**). Neither this variation nor additional exchange of the thioether by an ether (**105**) abrogated 5-LO inhibition (IC$_{50}$ = 0.8 and 0.6 µM). Elimination of the heteroatom between the benzene scaffold and the carboxylic moiety (**106**), in contrast, reduced the activity of the compounds (IC$_{50}$ = 5.0 µM). Shifting of the phenethoxy moieties to 3,4- (**107**) or 2,3- (**108**) positions led to similar inhibitory effects but an IC$_{50}$ of 0.9 µM was measured for the 2,5-bis-

phenethoxy substituted compound (**110**). 2,4-substitution (**109**), in contrast, resulted in a complete loss of function. Moreover, neither exchange of one nor of both phenylethoxy moieties of **106** to cyclohexylethoxy groups (**111**, **112** and **113**) led to IC_{50} values below 5 µM. Notably, introduction of a double bond linking the carboxylic group with the lipophilic backbone restored 5-LO suppression with an IC_{50} of 0.7 µM for the bis-phenethoxy substituted compound **114**. It was possible to maintain the potent inhibitory effect if the two phenethoxy moieties were rearranged from 3,5- to 2,5- (**115**) or 2,3- (**116**) positions and also a 2,5-bis-(2-cyclohexylethoxy) substituted derivative (**117**) was active. The inhibitory potency was even increased upon introduction of trifluormethyl groups in para-position of both phenethoxy moieties (**118**; IC_{50} = 0.3 µM). Finally, the ethylene spacers of **118** could be shortened to methylene without a loss of efficacy (**119**) and also exchange of one of the ((4-trifluormethyl)-benzyl)-oxy residues against a 2-cyclohexylethoxy moiety only slightly reduced the 5-LO inhibitory effect.

In analogy to the PA derivatives in **Tables 1-4**, also the compounds presented in this section were 2-10 fold less efficient to inhibit purified 5-LO, and the pyrimidine core seems to be important for a direct inhibitory effect on the enzyme.

Table 5:

comp.	structure	**5-LO product formation** IC$_{50}$ [µM] or *remaining activity (± S.E.) at 10 µM* PMNL	purif. 5-LO
104		0.8	7.2
105		0.6	*60.0 % (± 15.0)*
106		5.0	*80.3 % (± 13.5)*
107		6.4	*54.0 % (± 5.7)*

comp.	structure	5-LO product formation IC$_{50}$ [µM] or *remaining activity (± S.E.) at 10 µM* PMNL	purif. 5-LO
108		2.7	6.8
109		n.i.	*85.6 % (± 4.4)*
110		0.9	*65.5 % (± 9.9)*
111		5.2	*52.4 % (± 3.1)*

comp.	structure	**5-LO product formation** IC$_{50}$ [µM] or *remaining activity (± S.E.) at 10 µM* PMNL	purif. 5-LO
112		5.4	*65.0 % (± 14.8)*
113		5.4	*68.3 % (± 9.1)*
114		0.7	*62.7 % (± 17.6)*
115		0.9	*78.2 % (± 9.4)*

comp.	structure	5-LO product formation IC_{50} [µM] or *remaining activity* *(± S.E.) at 10 µM* PMNL	purif. 5-LO
116		0.8	*91.3 % (± 10.5)*
117		0.8[a]	6.0
118		0.3[a]	*55.6 % (± 2.7)*

comp.	structure	5-LO product formation IC$_{50}$ [µM] or *remaining activity (± S.E.) at 10 µM* PMNL	purif. 5-LO
119	CF$_3$ O O OH O CF$_3$	0.25[a]	*73.0 % (± 10.6)*
120	CF$_3$ O O OH O	0.4[a]	*55.5 % (± 7.9)*

[a]Data was performed by B. Jazzar or S. Luderer in the lab of Prof. Dr. O. Werz (University of Tuebingen).

S.E. = standard error

5.1.2. 2-(Phenylthio)hexanoic acid derivatives and analogs as 5-LO inhibitors

In order to further develop potent PPAR agonists structurally different from PA, our collaborating group of Prof. Dr. M. Schubert-Zsilavecz synthesized a series of 2-(phenylthio)hexanoic acid derivatives that were expected to be active on 5-LO as well. In brief, the beneficial 2-mercapto-hexanoic acid part of PA derivatives was linked to a phenolic backbone, etherified with a phenoxypropyl moiety. A variety of substituents were introduced at the terminal phenyl group to reveal SARs at this site of the molecule [244] (**Table 6**). Thus, an *ortho*-xylene moiety (**122**) increased the inhibitory effect on 5-LO 3-fold compared to the unsubstituted compound (**121**). No clear preference for one of the enantiomers of **122** could be detected. The remaining 5-LO activity obtained in presence of **123** shows that upon removal of the alkyl chain in α-position of the carboxylic group, the inhibitory effect on 5-LO product formation was attenuated. Insertion of a 2-methylphenyl- (**124**), 3-methylphenyl- (**125**) or 4-methylphenyl residue (**125**) led to slightly increased IC_{50} values between 4.8 µM and 5 µM. The same potency was obtained for the 3-methoxyphenyl substituted derivative (**128**) whereas introduction of a 2-methoxyphenyl- (**127**) or a 2-methoxy-4-chlorophenyl moiety (**129**) was detrimental. In contrast, bulky lipophilic substituents at the terminal phenyl group such as isopropyl (**130** and **131**), trifluormethyl (**132**) or 3-triflourmethyl-4-chlorophenyl (**133**) were helpful for 5-LO suppression (IC_{50} = 1.4-1.6 µM). Replacement of the phenyl by tetraline (**134**) was beneficial as well, but as observed for PA derivatives, the compound became inactive upon esterification. Similarly to their quality as substituents at PA, biphenylic (**136**) and quinoline (**139**) moieties led to highly potent inhibition of 5-LO product synthesis by the respective thiophenols. Again, it was found that biphenylic compounds were more effective in their acidic form and compounds with a quinoline residue turned out to inhibit cellular 5-LO product formation more efficiently than 5-LO in cell-free assays. Finally, exchange of the sulfur against carbon (**141**) or introduction of a double bond at this position (**142**) was tolerated without loss of activity as long as the α-butyl moiety was kept in the molecule.

Table 6 inhibition of 5-LO product formation by 2-(phenylthio)hexanoic acid derivatives:

comp.	X	R1	R2	R3	**5-LO product formation** IC_{50} [µM] or *remaining activity (± S.E.) at 10 µM* PMNL	purif. 5-LO
			R2–O–(CH2)3–O–C6H4–X–CH(R1)–C(=O)–O–R3			
121	S	CH_3		-H	9.5	*69.4 % (± 4.8)*
122	S	CH_3	CH_3 CH_3	-H	3.0	8.8
122a	S	CH_3	CH_3 CH_3	-H	1.1	10.0
122b	S	CH_3	CH_3 CH_3	-H	2.0	8.2
123	S	-H	CH_3 CH_3	-H	*69.9 % (± 9.8)*	*72.4 % (± 4.0)*
124	S	CH_3	CH_3	-H	4.8	*58.3 % (± 6.8)*
125	S	CH_3	CH_3	-H	4.8	*79.3 % (± 10.6)*
126	S	CH_3	H_3C	-H	5.0	*67.6 % (± 11.7)*

comp.	X	R1	R2	R3	5-LO product formation IC$_{50}$ [µM] or *remaining activity (± S.E.) at 10 µM* PMNL	purif. 5-LO
127	S	CH_3	OMe	-H	*57.4 % (± 11.9)*	*79.4 % (± 6.4)*
128	S	CH_3	OMe	-H	4.8	*67.7 % (± 5.8)*
129	S	CH_3	Cl, OMe	-H	*79.3 % (± 14.0)*	*59.2 % (± 7.4)*
130	S	CH_3	CH_3, CH_3	-H	1.6	7.8
131	S	CH_3	H_3C, CH_3	-H	1.4	6.0
132	S	CH_3	CF_3	-H	1.6	10.0
133	S	CH_3	CF_3, Cl	-H	1.5	2.0

comp.	X	R1	R2	R3	**5-LO product formation** IC$_{50}$ [µM] or *remaining activity (± S.E.) at 10 µM* PMNL	purif. 5-LO
134	S	CH_3		-H	1.4	4.5
135	S	CH_3		$-C_2H_5$	*87.4 % (± 2.8)*	*79.4 % (± 28.4)*
136	S	CH_3		-H	0.9	3.5
137	S	CH_3		$-C_2H_5$	*91.5 % (± 0.9)*	*83.0 % (± 38.7)*
138	S	CH_3	N	-H	*92.2 % (± 3.4)*	*59.0 % (± 12.7)*
139	S	CH_3	N	$-C_2H_5$	0.8	*68.3 % (± 12.8)*
140	C	-H	CH_3 CH_3	-H	*80.0 % (± 2.0)*	*81.8 % (± 14.4)*
141	C	CH_3	CH_3 CH_3	-H	2.0	*70.8 % (± 5.0)*

comp.	**structure**	**5-LO product formation** IC$_{50}$ [µM] or *remaining activity (± S.E.) at 10 µM*	
		PMNL	purif. 5-LO
142		1.6	*88.9 % (± 2.7)*

5.2. Biological evaluation of selected compounds as 5-LO inhibitors

Strategies to inhibit cellular 5-LO product formation can be a direct inhibition of the enzyme but also, for example, interference with AA release or FLAP [200]. Direct 5-LO inhibitors are categorized into redox-active compounds, iron ligand inhibitors, nonredox-type inhibitors and a fourth class of compounds that are supposed to act in an allosteric manner [262]. As shown above, 5-LO inhibition by acidic PA derivatives and 2-(phenylthio)hexanoic acid derivatives was more efficient in intact cells than in cell-free assays. Moreover, the esterified compounds do not suppress 5-LO directly, but if they possess heteroaromatic (e.g. quinolines) or not too bulky moieties, they are able to impair cellular 5-LO product formation quite efficiently. To further investigate the underlying mechanism of the 5-LO inhibitory effect of the various types of PA derivatives, mechanistic studies were performed with four distinct representatives from our SAR investigations (**Fig. 16**). Thus, the molecular pharmacological profile of the compounds was evaluated in various cell-based and cell-free assays with special experimental settings.

Since biphenylic PA derivatives showed a high efficacy on 5-LO, compound **39** as a very potent but simply structured molecule was chosen for biological characterization. Compound **25** served as a representative for the quinolinic PA derivatives that possess an increased potency upon esterification but do not inhibit isolated 5-LO. When cellular and cell-free assays are taken into account, the α-hexyl substituted 2-(bis-(phenethoxy)-pyrimidine-2-ylthio)-hexananoic acid **86** showed the best inhibitory profile of its sub-group and was therefore included in the molecularpharmacological studies. The fourth compound **136** was the most potent direct 5-LO inhibitor among the 2-(phenylthio)hexanoic acid derivatives.

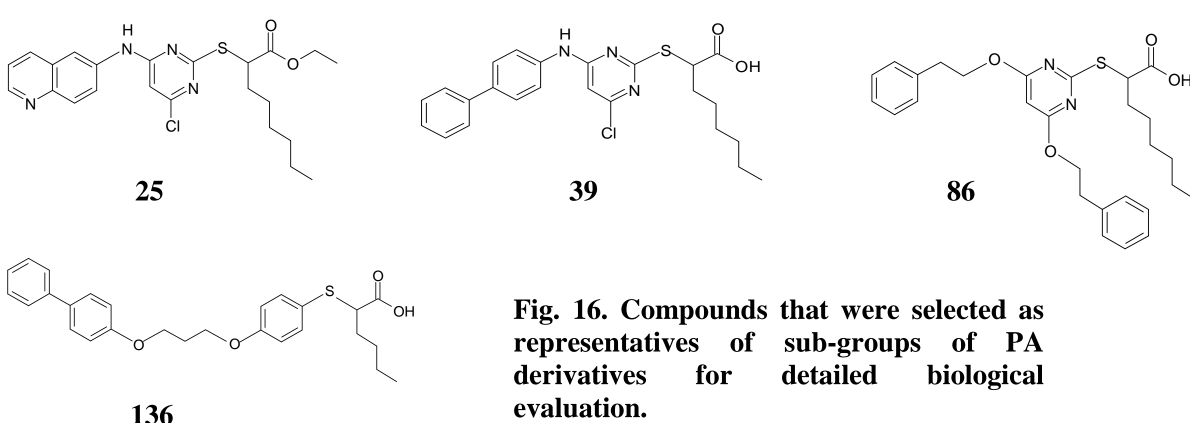
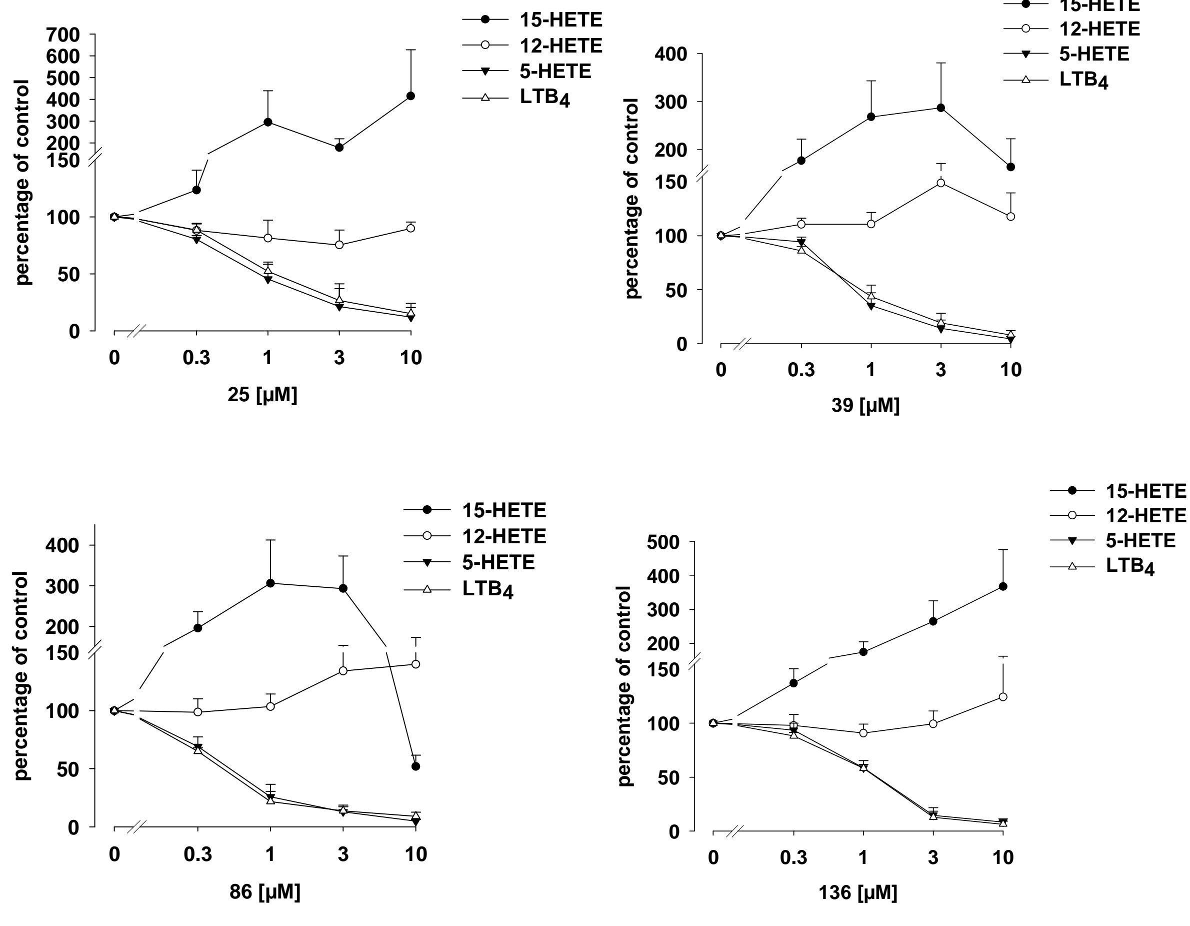


Fig. 16. Compounds that were selected as representatives of sub-groups of PA derivatives for detailed biological evaluation.

5.2.1. Inhibition of 5-LO product formation by the compounds in cell-based assays

5.2.1.1. Selectivity of the compounds for 5-LO towards other LOs

In order to assess the selectivity of the compounds for 5-LO compared to other LOs, suppression of product formation from 12/15-LO (15-LO-1), expressed in eosinophilic granulocytes, and from platelet-type-12-LO (present in PMNL-adherent platelets) was monitored. As shown in **Fig. 17**, the amount of 15-H(p)ETE was not decreased upon treatment with **25**, **39**, **136** or the control inhibitor BWA4C, and only moderately reduced by compound **86** at 10 µM. 12-H(p)ETE formation was also rather increased by the compounds, whereas BWA4C caused a weak suppression of the 12-LO product. 5-HETE and LTB_4 formation were efficiently blocked by all tested compounds.



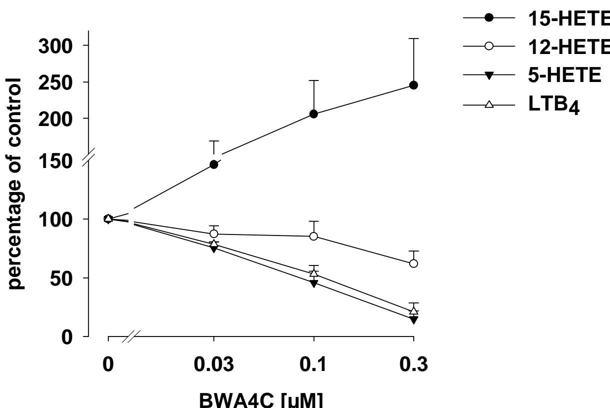


Fig. 17. Influence of the compounds on 5-LO, 12-LO and 15-LO product formation in ionophore A23187-stimulated PMNL. PMNL (5×10^6) were pre-incubated with compounds for 15 min at 37 °C. Then, 20 µM AA plus 2.5 µM ionophore A23187 were added and the reaction was stopped after 10 min. The amounts of 15-HETE, 12-HETE, 5-HETE and LTB_4 were determined by HPLC after 10 min incubation at 37 °C. Data are means + S.E., n = 4.

5.2.1.2. Influence of the stimulus on 5-LO inhibition by the compounds

It was demonstrated in previous studies that the potency of certain 5-LO inhibitors strongly depends on the applied stimulus used to evoke cellular 5-LO product synthesis [153],[154]. Stimulation of PMNL with the non-physiological ionophore A23187 leads to a strong Ca^{2+}-influx into the cell, whereas fMLP (a more physiologically relevant stimulus) activates cellular 5-LO via GPCR signaling leading to elevated $[Ca^{2+}]_i$ and phosphorylations of 5-LO [81]. The amount of 5-LO products in PMNL stimulated with fMLP is much lower than in cells activated with A23187 (< 1 ng/10^6 cells) [124]. Therefore, LTB_4 formation under these assay conditions was determined by an EIA. Compound **25** (tested by Dr. C. Pergola in our group) and compound **39** suppressed 5-LO product formation in PMNL stimulated with fMLP upon priming with LPS and Ada [144] with the same potency as upon ionophore A23187 stimulation (F**ig. 18**). In contrast, the IC_{50} values of **86** and **136** were slightly augmented upon stimulation of the cells with LPS/fMLP.

Genotoxic or hyperosmotic cell stress leads to phosphorylation of 5-LO by p38 MAPK-dependent MK-2 accompanied by an increase in LT biosynthesis. The efficacy of some nonredox-type 5-LO inhibitors (e.g. ZM 230487) is attenuated under these conditions [249]. When PMNL were exposed to 300 mM NaCl plus 40 µM AA, a decreased potency of all four test compounds was evident but curiously also for the iron-ligand inhibitor BWA4C (**Fig. 19**). However, this effect was less pronounced for BWA4C and **136** than for the other derivatives and the positive control ZM230487.

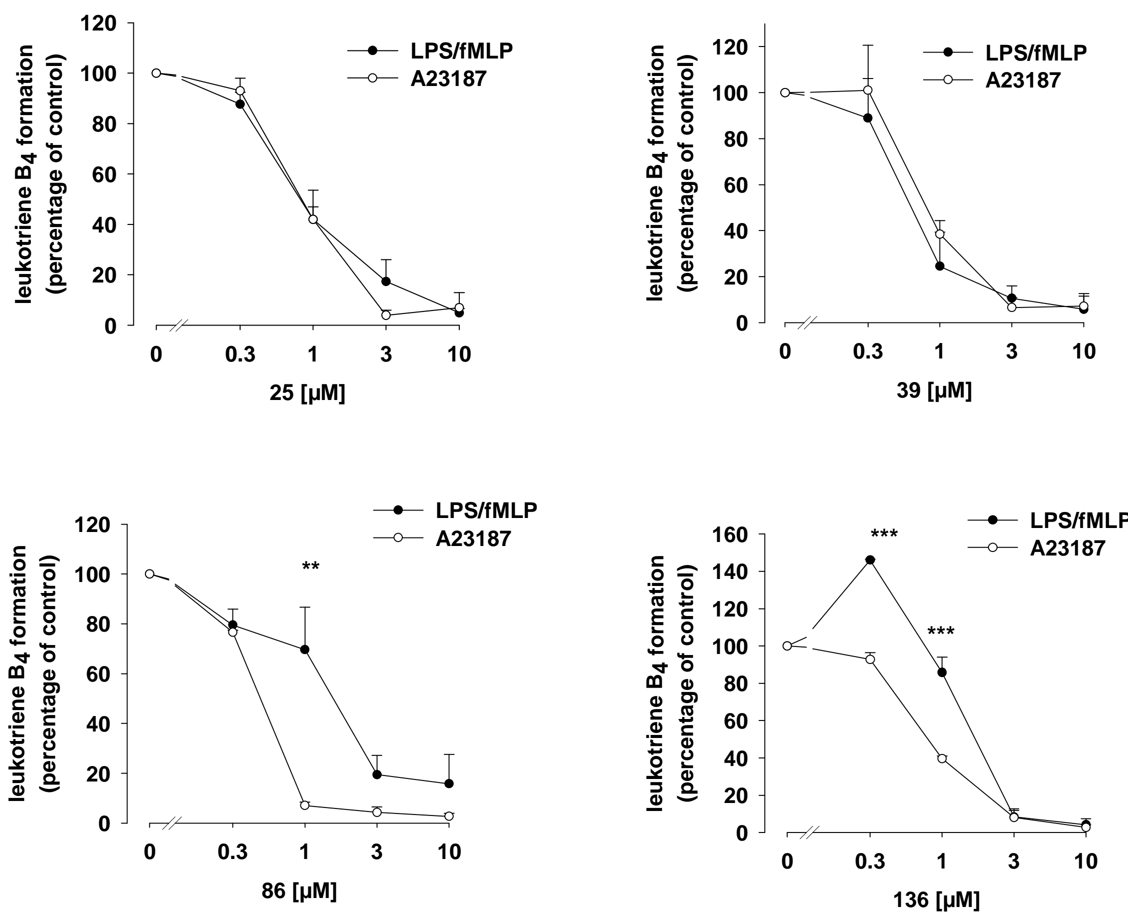


Fig. 18. 5-LO inhibition by the compounds in PMNL stimulated with LPS/fMLP. PMNL (5×10^6) were pre-incubated with compounds for 15 min at 37 °C, and 2.5 µM ionophore A23187 was added. LTB_4 was determined by HPLC after 10 min incubation at 37 °C. Alternatively, PMNL (2×10^7) were primed with 1 µg/ml LPS and then stimulated with 1 µM fMLP to induce 5-LO product formation. After 5 min, the amount of released LTB_4 was determined by EIA. Data are means + S.E., n = 4. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. samples that were stimulated with ionophore A23187 at corresponding concentrations of test compounds, ANOVA + Tukey post hoc test.

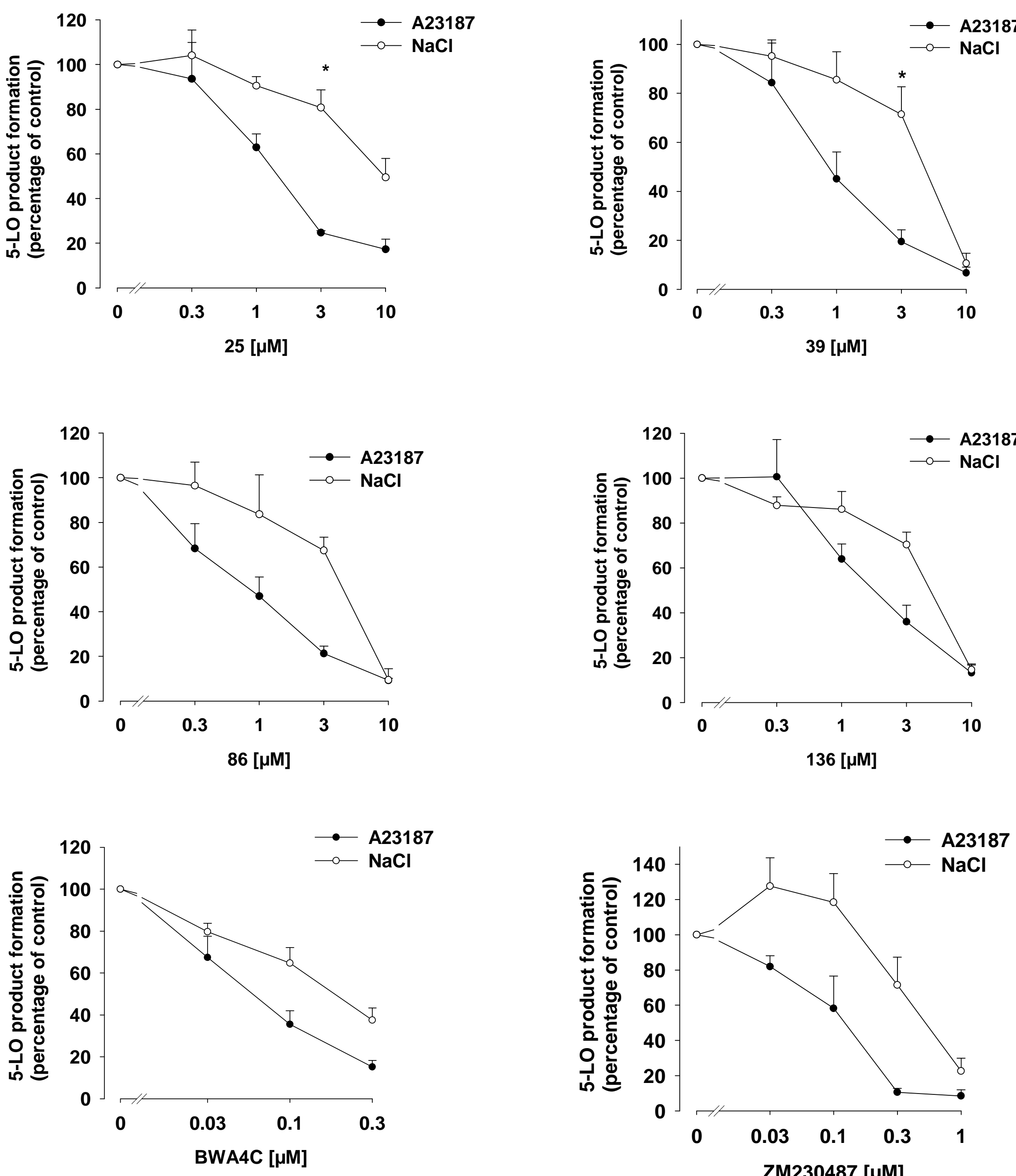


Fig. 19. Efficiency of the compounds under conditions that activate 5-LO by cell-stress. PMNL (5×10^6) were pre-incubated with compounds for 15 min at 37 °C. NaCl (0.3 M) was supplemented 3 min before addition of 40 µM AA. Alternatively, cells were stimulated with 2.5 µM ionophore A23187 in presence of 40 µM AA. After 10 min at 37 °C, the reaction was stopped and 5-LO products were measured. Data are means + S.E., n = 4. *p < 0.05 vs. samples that were stimulated with ionophore A23187 at corresponding concentrations of test compounds, ANOVA + Tukey post hoc test.

5.2.1.3. Influence of the AA concentration on cellular 5-LO inhibition by the compounds

The substrate concentration is a critical parameter for 5-LO inhibition by so-called competitive 5-LO inhibitors or by FLAP antagonists [165], [153]. Addition of 20 µM AA together with ionophore A23187 rather improved the efficiency of **25** and **39** in human PMNL as compared to their inhibitory effect upon application of ionophore A23187 alone **(Fig. 20)**. Almost no discrepancy in the potency was observed for **86** and **136**. However, a loss of activity of all four compounds occurred at AA concentrations ≥ 40 µM (which are not considered physiological). Suppression of 5-LO products by BWA4C was slightly reduced when 20 µM AA were added together with ionophore A23187 but was not further decreased with higher substrate concentrations (40 or 60 µM AA). Since addition of moderate AA concentrations does not abrogate the inhibitory effect of the compounds on 5-LO product formation, interference with FLAP seems rather unlikely. This is supported by the fact that equal IC_{50} values were obtained in PMNL from male and female donors (not shown), whereas FLAP inhibitors are more potent in female PMNL (Pergola et. al., unpublished data).

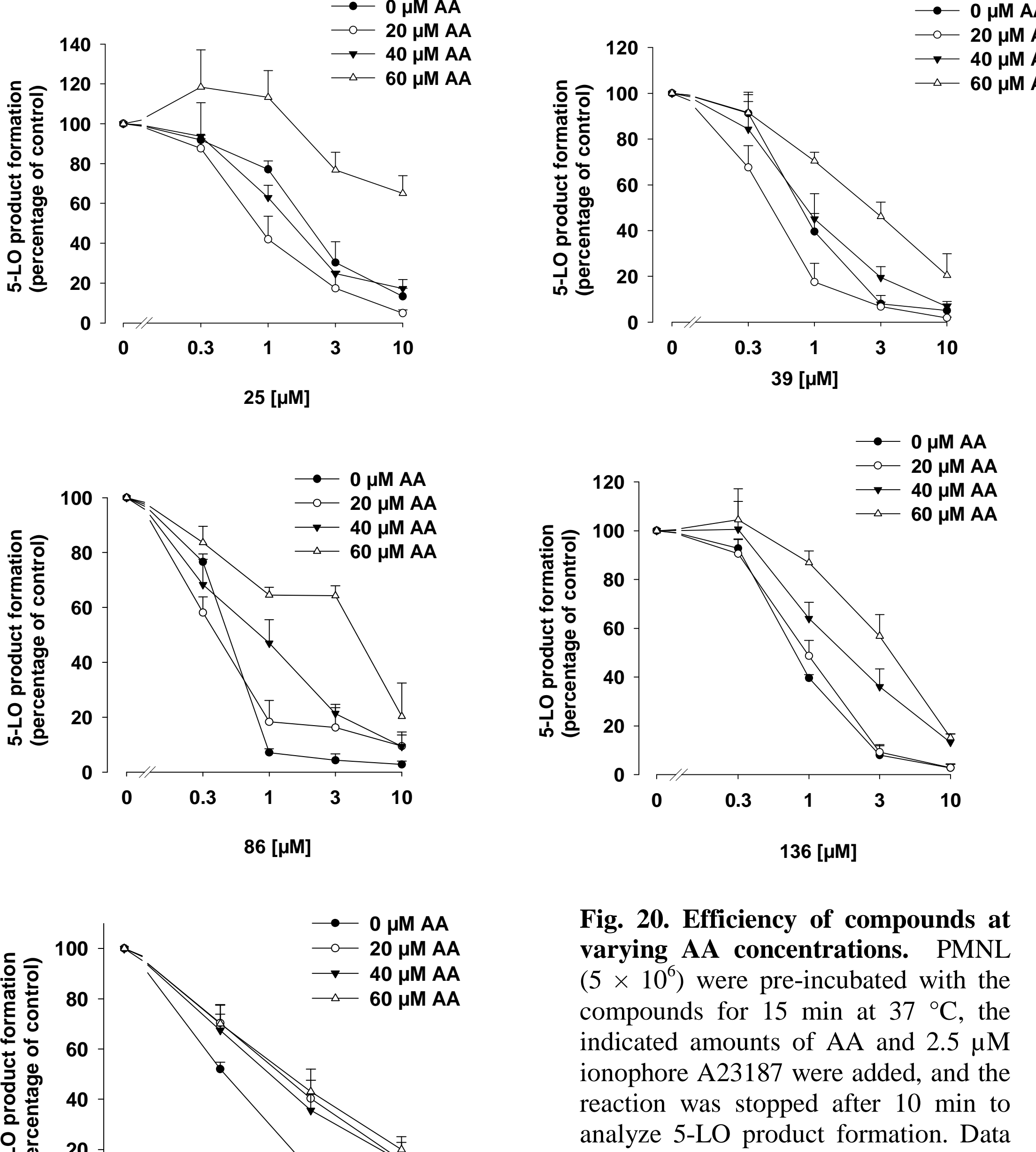


Fig. 20. Efficiency of compounds at varying AA concentrations. PMNL (5×10^6) were pre-incubated with the compounds for 15 min at 37 °C, the indicated amounts of AA and 2.5 µM ionophore A23187 were added, and the reaction was stopped after 10 min to analyze 5-LO product formation. Data are means + S.E., n = 3.

5.2.1.4. Influence of the compounds on 5-LO translocation to the nuclear membrane

Stimulation of PMNL from female donors leads to a redistribution of 5-LO from the cytosol to the nuclear membrane [144], and it was demonstrated that LT formation is efficiently blocked by several agents that inhibit 5-LO translocation such as FLAP inhibitors [165], hyperforin [210] or sulindac sulfide [215]. The effects of **25**, **39**, **86** and **136** on the subcellular localization of 5-LO were assessed by means of a crude cell fractionation using mild detergent lysis (0.1% NP-40) to separate the nuclear from the non-nuclear fraction. Interestingly, a concentration-dependent 5-LO accumulation at the nucleus was observed if PMNL from females were preincubated with any of the test compounds and subsequently stimulated with 2.5 µM ionophore A23187. In contrast, hyperforin (30 µM) prevented accumulation of 5-LO in the nuclear fraction as expected (**Fig. 21**).

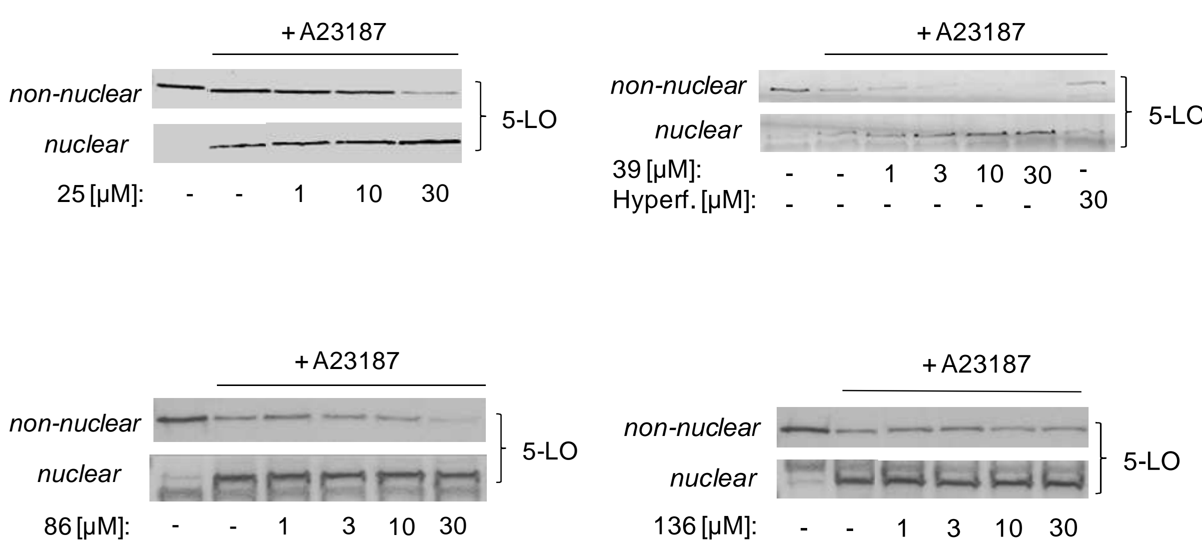


Fig. 21. Influence of the compounds on 5-LO translocation in PMNL stimulated with ionophore A23187 and separated into nuclear and non-nuclear fractions. Freshly isolated PMNL (3×10^7) were pre-incubated with the compounds or with vehicle (DMSO) for 15 min at 37 °C. 2.5 µM ionophore A23187 was added to the samples as indicated, and cells were incubated for another 5 min at 37 °C. 5-LO was detected in nuclear and non-nuclear fractions by immunoblotting after subcellular fractionation using 0.1% NP-40. Similar results were obtained in two additional independent experiments.

Since **39** provoked the most prominent shift of 5-LO to the nucleus, this compound was chosen for further studies on 5-LO translocation. As **Fig. 22** shows, increased amounts of nuclear 5-LO upon treatment with **39** were also found after priming of female PMNL with LPS (1 µg/ml) and Ada (0.3 U/ml) followed by stimulation with fMLP (1 µM).

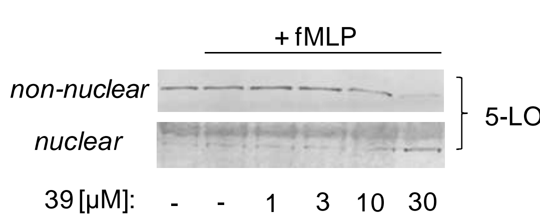


Fig. 22. Influence of compound 39 on 5-LO translocation in PMNL stimulated with LPS/fMLP and divided into nuclear and non-nuclear fractions. PMNL (3 × 10^7) were primed with LPS (1 µg/ml) and Ada (0.3 U/ml) followed by stimulation with fMLP (1 µM) for 5 min at 37 °C. 5-LO was detected in nuclear and non-nuclear fractions by immunoblotting after subcellular fractionation using 0.1% NP-40. Similar results were obtained in two additional independent experiments.

To evaluate if **39** itself is able to induce 5-LO translocation in resting female PMNL, cells were treated with the compound for 20 min at 37 °C in presence of Ada but without being exposed to another stimulus. Indeed, **39** caused a distinct increase of 5-LO in the nuclear fraction (**Fig. 23**). Notably, **40** the inactive ethyl ester of **39** had no influence on 5-LO distribution so that the acidic group seems to be important for perinuclear 5-LO redistribution. As expected, hyperforin did not provoke nuclear localization of 5-LO in unstimulated cells.

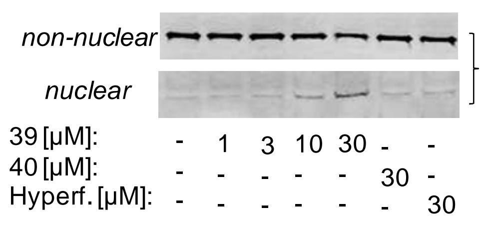


Fig. 23. Influence of compound 39 on 5-LO translocation in resting PMNL. Freshly isolated PMNL (3 × 10^7) were incubated with **39** at the indicated concentrations, **40** (30 µM), hyperforin (30 µM) or vehicle (DMSO) in presence of 0.3 U Ada for 20 min at 37 °C. 5-LO was detected in nuclear and non-nuclear fractions by immunoblotting after subcellular fractionation using 0.1% NP-40. Similar results were obtained in two additional independent experiments.

To investigate if **39** indeed targets 5-LO to a membrane structure (and not to the nucleosol), PMNL were completely disrupted by sonification after incubation with **39**, and separated into a soluble and a membraneous fraction by 100,000×g centrifugation. **39** directs 5-LO to the membrane compartment, regardless whether or not ionophore A23187 is added as stimulus (**Fig. 24**). Interestingly, hyperforin led to a strong accumulation of 5-LO in the membrane fraction. As this accumulation was not observed after cell fractionation with NP-40 one may assume that hyperforin redirects 5-LO from the nuclear membrane to the cellular membrane.

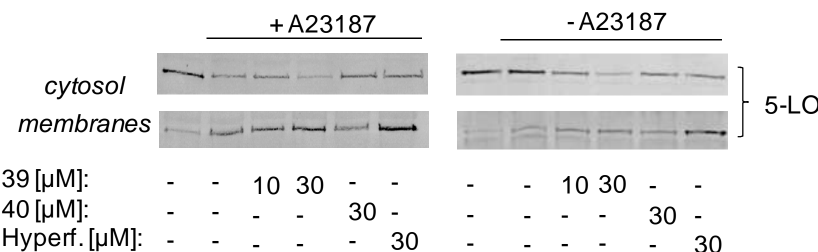


Fig. 24. Influence of compound 39 on 5-LO translocation in PMNL; separation of cytosol and membranes. Freshly isolated PMNL (3×10^7) were treated as described in **Fig. 21 or Fig. 23, respectively**. Subcellular localization of 5-LO was determined by immunoblotting after sonication of the cells and preparation of soluble (S100) and membrane (P100) fractions by 100,000×g centrifugation. Similar results were obtained in two additional independent experiments.

Finally, indirect immunofluorescence (IF) studies were intended in order to investigate "in cell" localization of 5-LO after treatment with the compounds. In brief, PMNL (1.5×10^6, freshly isolated from buffy coats of female donors) treated with **39** (10 µM or 30 µM) for 15 min were spun on poly-lysine coated coverslips and subsequently stimulated with ionophore A23187. After fixation of the cells with methanol, subcellular localization of 5-LO can be detected with a Zeiss Axiovert 200 M microscope by use of an anti-5-LO antibody followed by application of a fluorescently labeled anti IgG against the first antibody. However, cells treated with **39** did no longer attach to the poly-lysine coated coverslips and could therefore not be analyzed by fluorescence microscopy. The ester of **39** (compound **40**) as well as hyperforin, in contrast, had no influence on the affinity of the cells towards the coverslips, indicating that **39** with its acidic function is somehow able to interfere with cellular structures.

5.2.2. Inhibition of 5-LO product formation by the compounds in cell-free assays

5.2.2.1. Reversibility of the 5-LO inhibitory effect

Most of the direct 5-LO inhibitors act in a reversible manner. Therefore, it was assessed whether inhibition of 5-LO product formation by the derivatives could be abrogated after dilution. Partially purified human recombinant 5-LO was pre-incubated with the indicated amounts of **39**, **86** and **136** (since **25** does not influence 5-LO directly, it was not considered in 5-LO assays with the partially purified enzyme). After 10-fold dilution with assay buffer and subsequent addition of 20 µM AA, 5-LO product formation was almost at the same level as after treatment with **39** or **86** without precedent dilution (**Fig. 25**), whereas the inhibitory effect **136** could not be fully reversed.

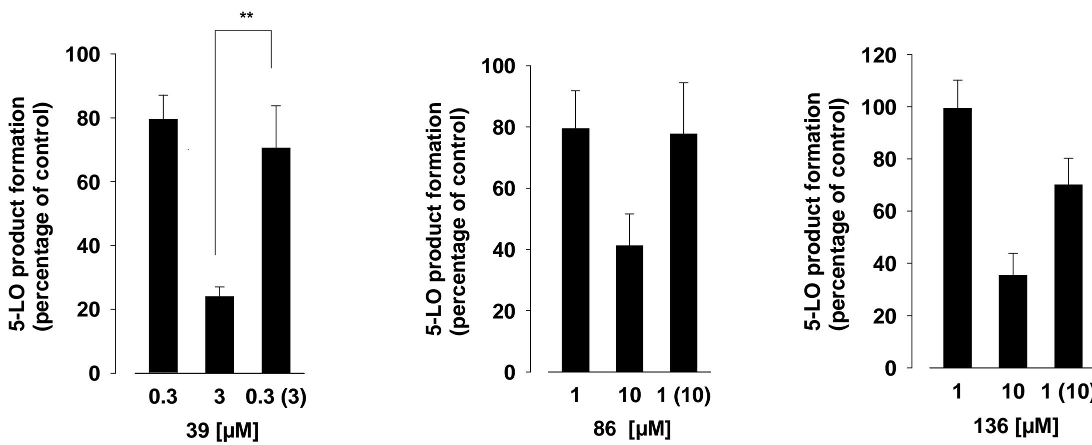


Fig. 25. Wash out experiments. 5-LO (0.5 µg) was pre-incubated with the indicated amount of test compounds for 10 min at 4 °C. Then, one aliquot was diluted with assay buffer ten-fold, whereas the other one was not altered, and 20 µM AA plus 1 mM Ca^{2+} were added to start the reaction. For comparison, purified 5-LO was pre-incubated with the corresponding higher compound concentration for 10 min at 4 °C and then, 20 µM AA plus 1 mM Ca^{2+} were added (no dilution). All samples were incubated for 10 min at 37 °C, and 5-LO product formation was analyzed as described. Values are given as means + S.E., n = 3, **p < 0.01 vs. inhibition w/o dilution, ANOVA + Bonferroni post hoc test.

5.2.2.2. Redox activity of the compounds

5-LO inhibitors of the redox-type attenuate 5-LO activity by reducing the active-site iron to the ferrous form so that the catalytic cycle of 5-LO is interrupted. To determine whether PA derivatives or 2-(phenylthio)hexanoic acid derivatives possess radical scavenging properties, the compounds were tested for their ability to reduce 1,1-diphenyl-2-picrylhydrazyl (DPPH). All four derivatives (applied in concentrations up to 100 µM) failed in this respect (**Fig. 26**), whereas the positive controls L-cysteine and ascorbic acid concentration-dependently reduced DPPH.

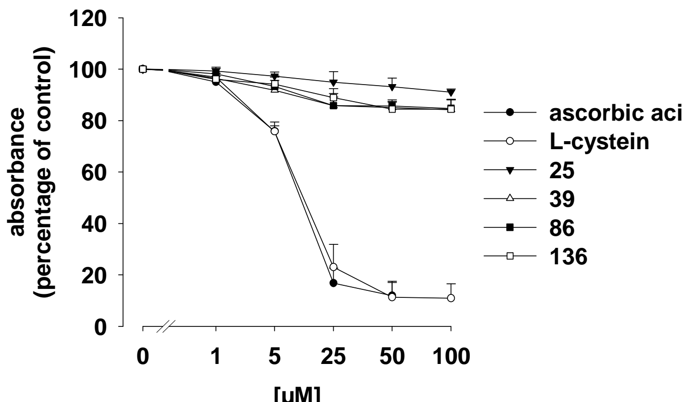


Fig. 26. Analysis of radical scavenging properties of 25, 39, 86 and 136. The indicated concentrations of the derivatives were incubated with 50 µM DPPH in EtOH for 30 min at RT, shaking. Reduction of the absorbance at 520 nm was measured with a multiwell scanning spectrophotometer. Values are given as means + S.E., n = 3.

5.2.2.3. Influence of the substrate concentration on cell-free 5-LO inhibition by the compounds

To test if the compounds suppress 5-LO equally efficient at different substrate concentrations, the amount of AA in the cell-free assay was altered. Upon addition of 7.5 µM AA or less, the IC_{50} values for **39** were determined at 7-8 µM, whereas a considerable shift in the efficacy was observed between 7.5 µM and 10 µM AA. Thus, substrate concentrations between 10 µM and 40 µM even yielded IC_{50} values of 1.3-1.5 µM (**Fig. 27**). Only at 60 µM AA, 5-LO inhibition by **39** was slightly impaired. Increments in the potency were also observed for **86** with the best 5-LO repression occurring at 20 or 30 µM AA, respectively. Finally, the inhibitory effect of **136** was enhanced with increasing substrate concentrations from 5-10-15-20-30 µM AA and moderately declined at 60 µM AA. In contrast to **39** and **86**, compound **136** was not able to suppress 5-LO

product formation completely, regardless of the applied amount of AA. The control inhibitor BWA4C suppressed 5-LO with consistent IC_{50} values at 5, 10, 20 and 60 µM AA.

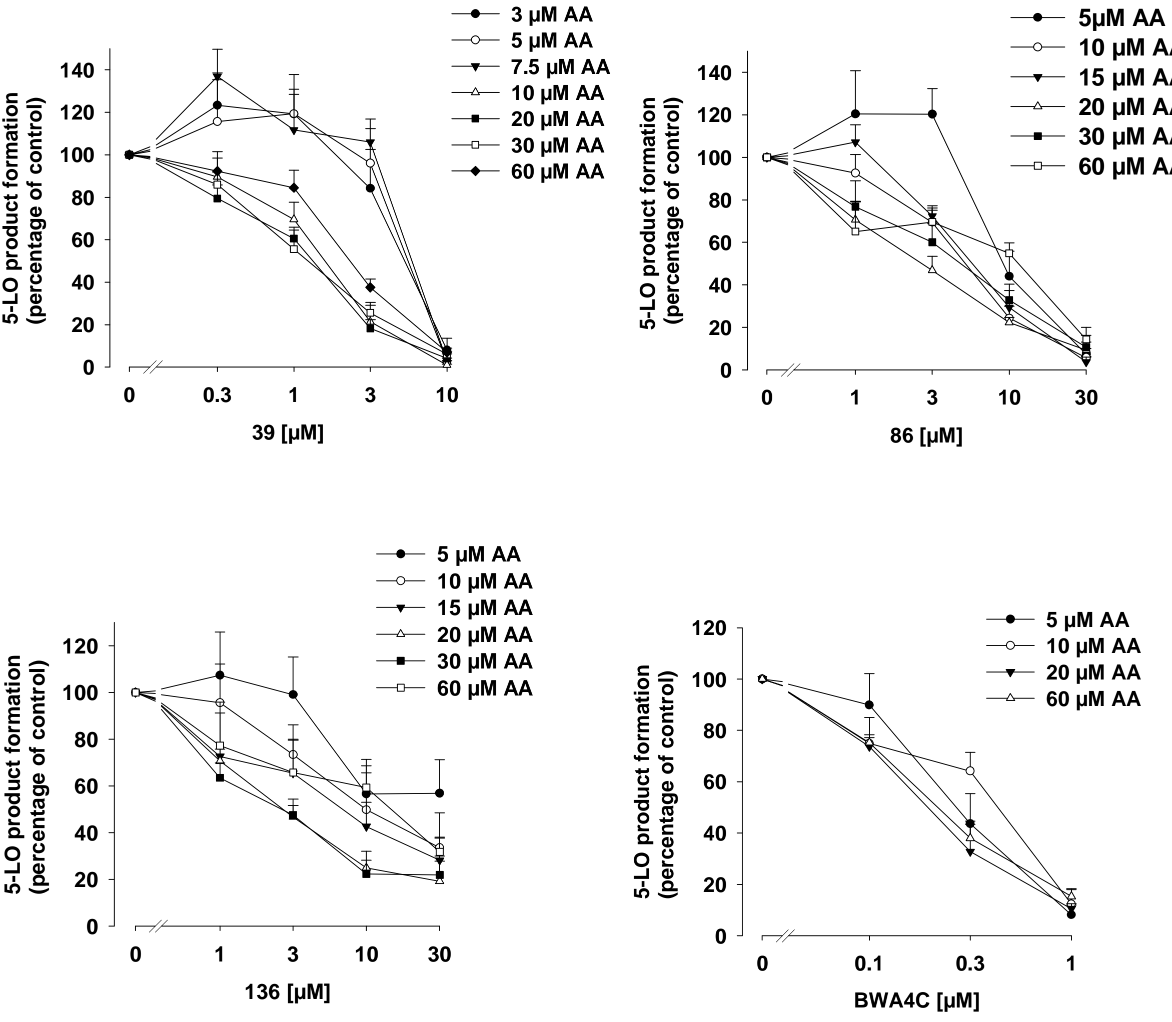
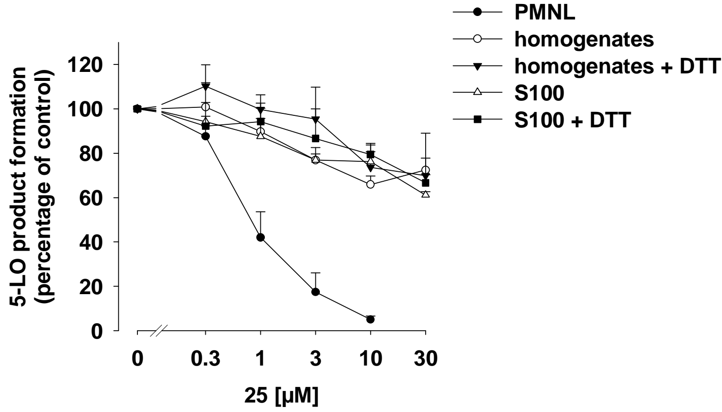
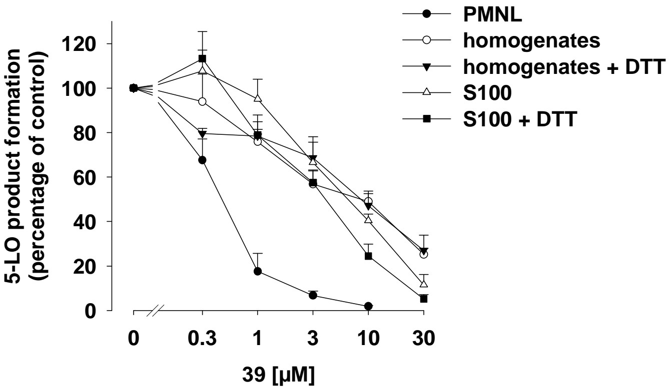


Fig. 27. Effect of the compounds on 5-LO product formation at different substrate concentrations. Partially purified human recombinant 5-LO (0.5 µg) expressed in *E.coli* was supplemented with 1 mM ATP and pre-incubated in 1 ml PBS buffer plus 1 mM EDTA with the compounds at the indicated concentrations or with vehicle (DMSO) for 5–10 min at 4 °C. Samples were pre-warmed at 37 °C for 30 sec, and 5-LO product formation was started by addition of 2 mM $CaCl_2$ together with the indicated amount of AA. After another 10 min, 5-LO product formation was determined. Values are given as mean + S.E., n ≥ 3.

5.2.2.4. Influence of the redox tone on 5-LO inhibition by PA derivatives and 2-(phenylthio)hexanoic acid derivatives

Most of the nonredox-type 5-LO inhibitors potently suppress LT formation in intact cells but fail in cell-free assays because they require reducing conditions [153]. Thus, an inhibitory effect of these substances on 5-LO product synthesis in homogenates from PMNL can only be attained upon addition of DTT (to reconstitute GPx activity). The efficacy of PA derivatives and 2-(phenylthio)hexanoic acid derivatives to inhibit partially purified 5-LO was also less pronounced than in intact PMNL (as shown above) and clearly reduced in homogenates from PMNL or their corresponding 100,000×g supernatants (S100) with IC_{50} values ≥ 10 µM, **Fig. 28**. However, addition of 1 mM DTT to homogenates or S100 did not restore the potency of the compounds. The control inhibitor BWA4C blocked 5-LO products in broken cell fractions with the same efficiency as in intact PMNL, regardless of the redox-tone.





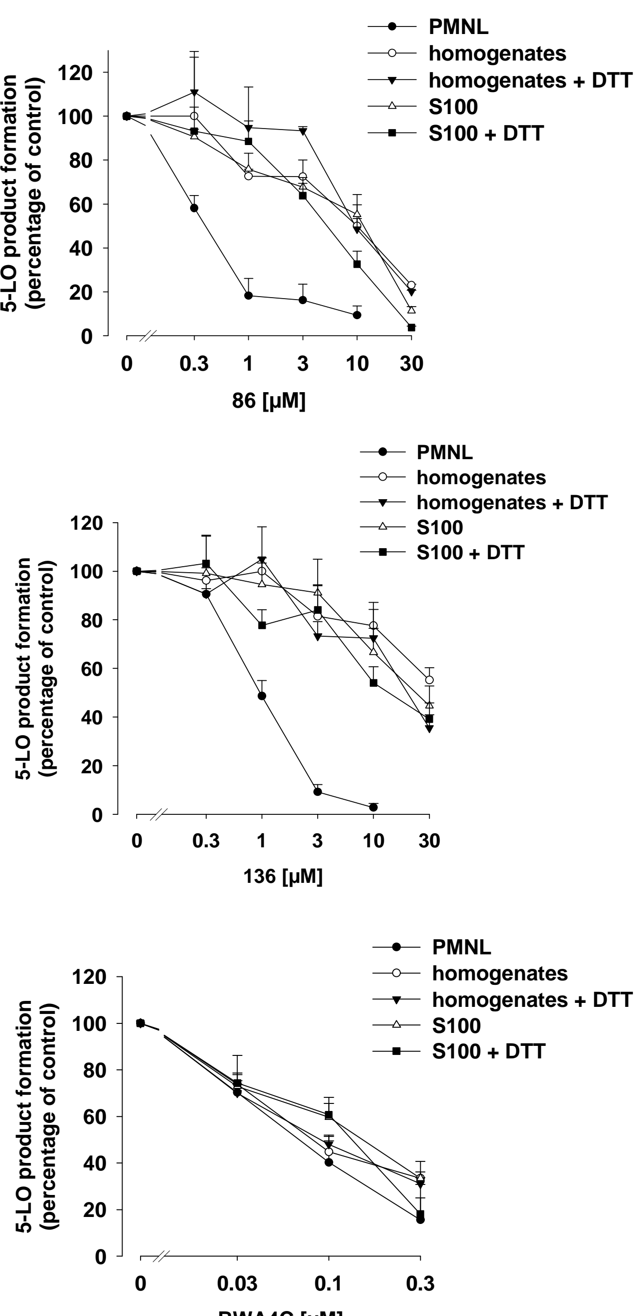


Fig. 28. Influence of DTT on the inhibition of 5-LO product formation by the compounds. Freshly isolated PMNL (5×10^6) were pre-incubated with compounds for 15 min at 37 °C. Then, 20 µM AA plus 2.5 µM ionophore A23187 were added and 5-LO product formation was determined after 10 min. Homogenates or S100 (corresponding to 5×10^6 PMNL, each) were diluted with PBS/EDTA. After addition of 1 mM ATP, samples were pre-incubated with the compounds at the indicated concentrations or with DMSO as vehicle for 5–10 min at 4 °C. Then, samples were pre-warmed at 37 °C for 30 s, and 5-LO product formation was started by addition of 2 mM $CaCl_2$ and 20 µM AA. After 10 min, 5-LO product formation was determined. Values are given as mean + S.E., n = 3 - 4.

In addition, elevation of the peroxide tone by inclusion of 1 µM 13(S)-HpODE in the cell-free assay did not affect the potency any of the compounds **(Fig. 29).**

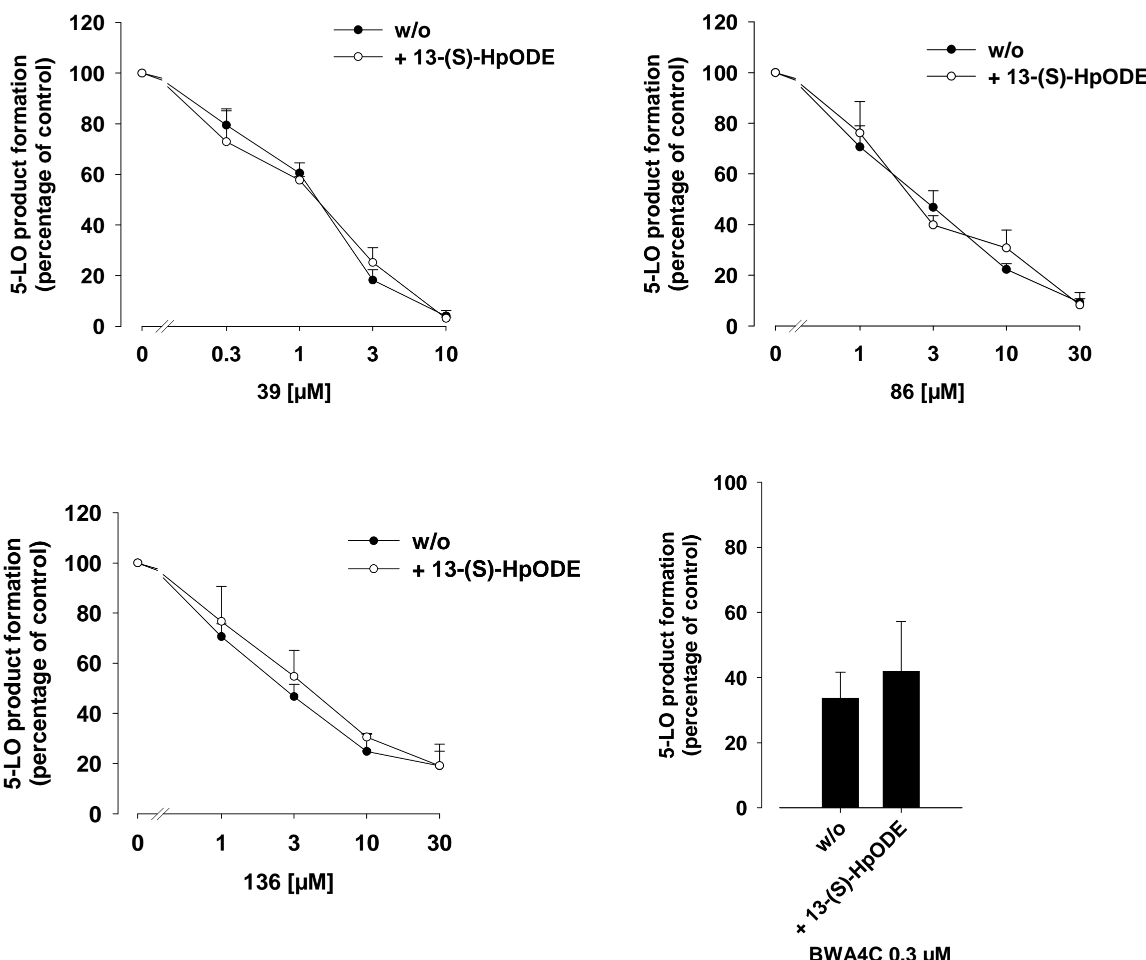
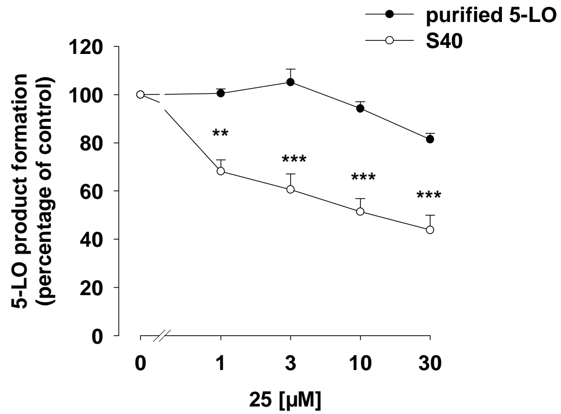
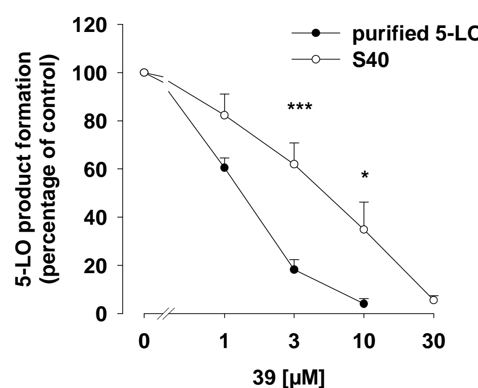


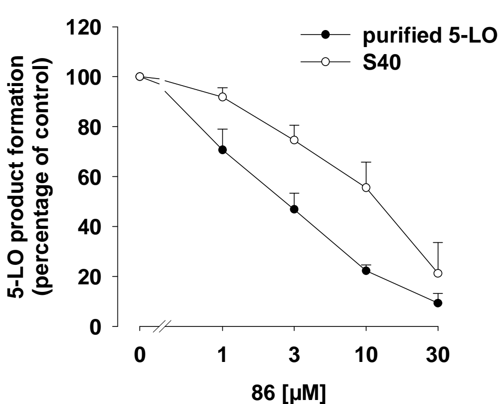
Fig. 29. Influence of LOOHs on 5-LO inhibition by the compounds. Partially purified human recombinant 5-LO (0.5 µg) expressed in *E.coli* was supplemented with 1 mM ATP and pre-incubated in 1 ml PBS buffer plus 1 mM EDTA with the compounds at the indicated concentrations or with vehicle (DMSO) for 5 – 10 min at 4 °C. Samples were pre-warmed at 37 °C for 30 sec, and 5-LO product formation was started by addition of 2 mM $CaCl_2$ plus 20 µM AA in presence or absence of 13(S)-HpODE (1 µM). After another 10 min, 5-LO product formation was determined. Values are given as mean + S.E., n = 3, ANOVA + Tukey post hoc test.

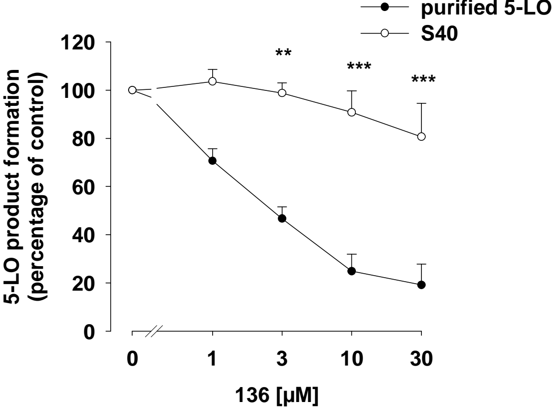
5.2.2.5. Influence of cellular components on 5-LO inhibition by the compounds

Interestingly, the potent 5-LO inhibition by **39**, **86** and **136** was not only impaired in homogenates from PMNL but also in the 40,000×g (S40) supernatant from *E. coli* lysates compared to partially purified 5-LO. **Fig. 30** shows that the IC_{50} values of **39** and **86** under these conditions were elevated about 5-fold, and for **136** this effect was even more pronounced (S40: 80.6% remaining activity at 30 µM of **136**). The efficacy of **25** in contrast, was increased if cellular components were present (S40: 43.8% remaining activity at 30 µM). Possibly, **25** is cleaved by esterases from *E. coli* resulting in a similar potency in S40 as its corresponding free acid **24** (52.7% remaining activity at 30 µM, not shown). Almost identical concentration-response curves for purified 5-LO and S40 were obtained with the control inhibitor BWA4C. Consequently, it seems that cellular components somehow attenuate the inhibitory effect of **39**, **86** and especially of **136** on 5-LO.









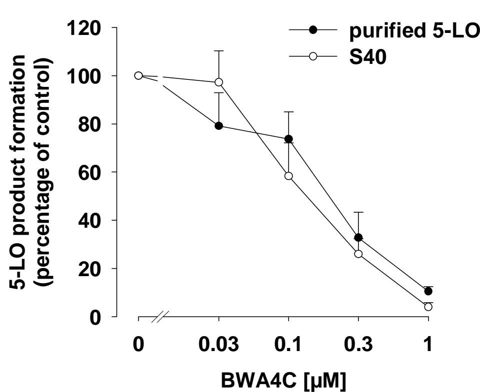
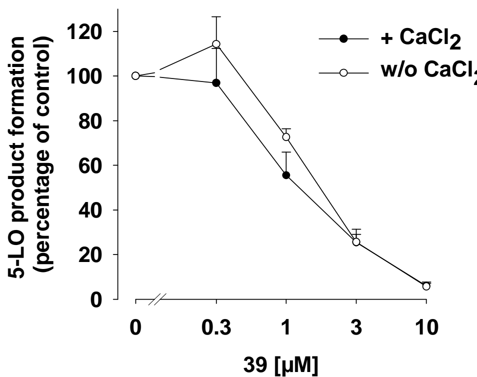
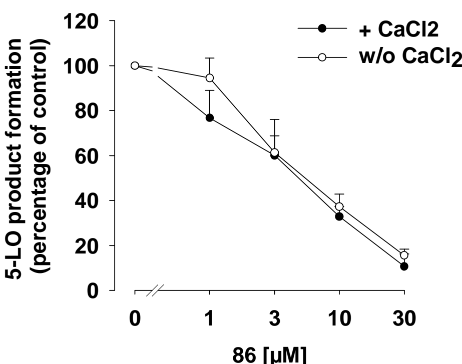


Fig. 30. 5-LO inhibition in S40 supernatants from E. coli. Aliquots of S40 supernatants from *E.coli* lysates (corresponding to 4 ml *E.coli* culture) or 0.5 µg partially purified 5-LO were diluted with PBS/EDTA. After addition of 1 mM ATP, samples were pre-incubated with the compounds at the indicated concentrations or with DMSO as vehicle for 5–10 min at 4 °C. Then, samples were pre-warmed at 37 °C for 30 s, and 5-LO product formation was started by addition of 2 mM $CaCl_2$ and 20 µM AA. After 10 min, 5-LO product formation was determined. Values are given as mean + S.E., n $\geq$ 3. *p < 0.05, **p < 0.01, ***p < 0.001 vs. inhibition of partially purified 5-LO at corresponding concentrations of test compounds, ANOVA + Tukey post hoc test.

5.2.2.6. Effects of 5-LO modulatory factors on the efficiency of the compounds

5-LO inhibition by PA derivatives and 2-(phenylthio)hexanoic acid derivatives might be compromised by factors that modulate 5-LO activity such as Ca^{2+}-ions, phospholipids or membranes, glycerides and CLP (all apparently acting via the C2-like domain) [66]. All four derivatives comparably inhibited partially purified 5-LO in the presence or absence of Ca^{2+} (**Fig. 31**), excluding a primary interference of the compounds with Ca^{2+}-binding residues within the C2-like domain.





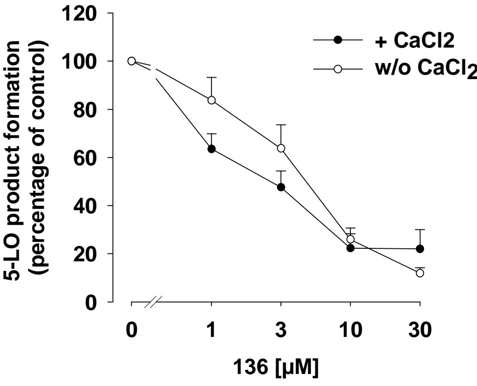


Fig. 31. Influence of Ca^{2+} on 5-LO inhibition by the compounds. Partially purified human recombinant 5-LO (0.5 µg) expressed in *E.coli* was supplemented with 1 mM ATP and pre-incubated in 1 ml PBS buffer plus 1 mM EDTA with the compounds at the indicated concentrations or with vehicle (DMSO) for 5–10 min at 4 °C. Samples were pre-warmed at 37 °C for 30 sec and 5-LO product formation was started by addition of 30 µM AA with or without Ca^{2+}. After another 10 min, 5-LO product formation was determined. Values are given as mean + S.E., n = 3, ANOVA + Tukey post hoc test.

Since PA derivatives and 2-(phenylthio)hexanoic acid derivatives are less efficient in homogenates than at isolated 5-LO, cellular components may attenuate their inhibitory potency. A similar effect was found for hyperforin in crude homogenates, and it was demonstrated that 5-LO inhibition by hyperforin is clearly reduced after addition of PC [210]. As **Fig. 32** shows, the efficacy of **39** to inhibit partially purified 5-LO is significantly attenuated in presence of PC (25 μg/ml) as well, whereas this effect is less prominent for **86** and **136**. Phosphatidylserine (PS) in contrast, hardly influenced the potency of the compounds. 5-LO inhibition by BWA4C was only marginally altered after addition of phospholipids.

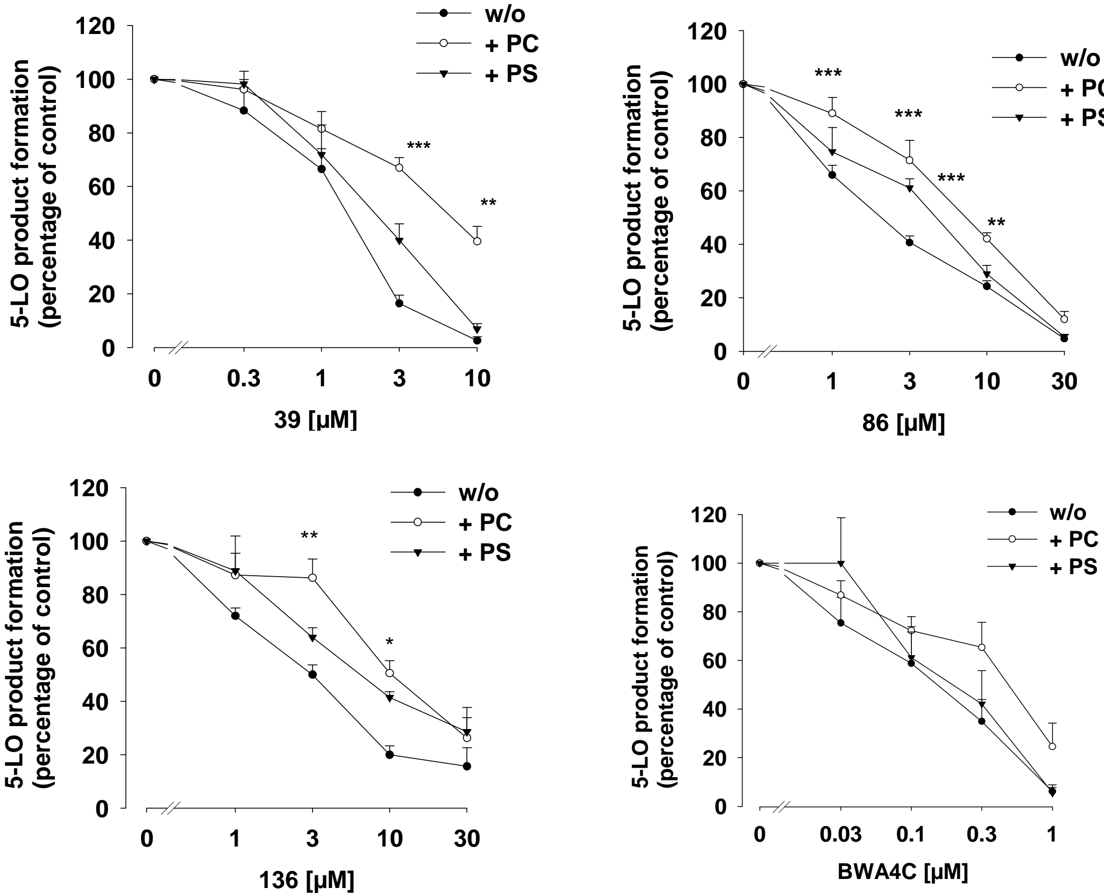


Fig. 32. Effect of phospholipids on 5-LO inhibition by the compounds. Partially purified human recombinant 5-LO (0.5 μg) expressed in *E.coli* was supplemented with 1 mM ATP and pre-incubated in 1 ml PBS buffer plus 1 mM EDTA with the compounds at the indicated concentrations or with vehicle (DMSO) for 5-10 min at 4 °C with or without PC or PS (25 μg/ml, each) as given in the figure. Samples were pre-warmed at 37 °C for 30 sec and 5-LO product formation was started by addition of 2 mM $CaCl_2$ plus 20 μM AA. After another 10 min, 5-LO product formation was determined. Values are given as mean + S.E., $n \geq 5$; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. samples without phospholipids (w/o) at corresponding concentrations of inhibitors, ANOVA + Tukey post hoc test.

5-LO inhibition of the compounds was not only altered in presence of PC but also upon addition of OAG. As **Fig. 33** shows, OAG (30 µM) clearly impaired the potency of all three derivatives, although the IC_{50} value of **39** was only marginally decreased. In agreement with previous data [210], the 5-LO inhibitory curve of BWA4C was not affected by OAG.

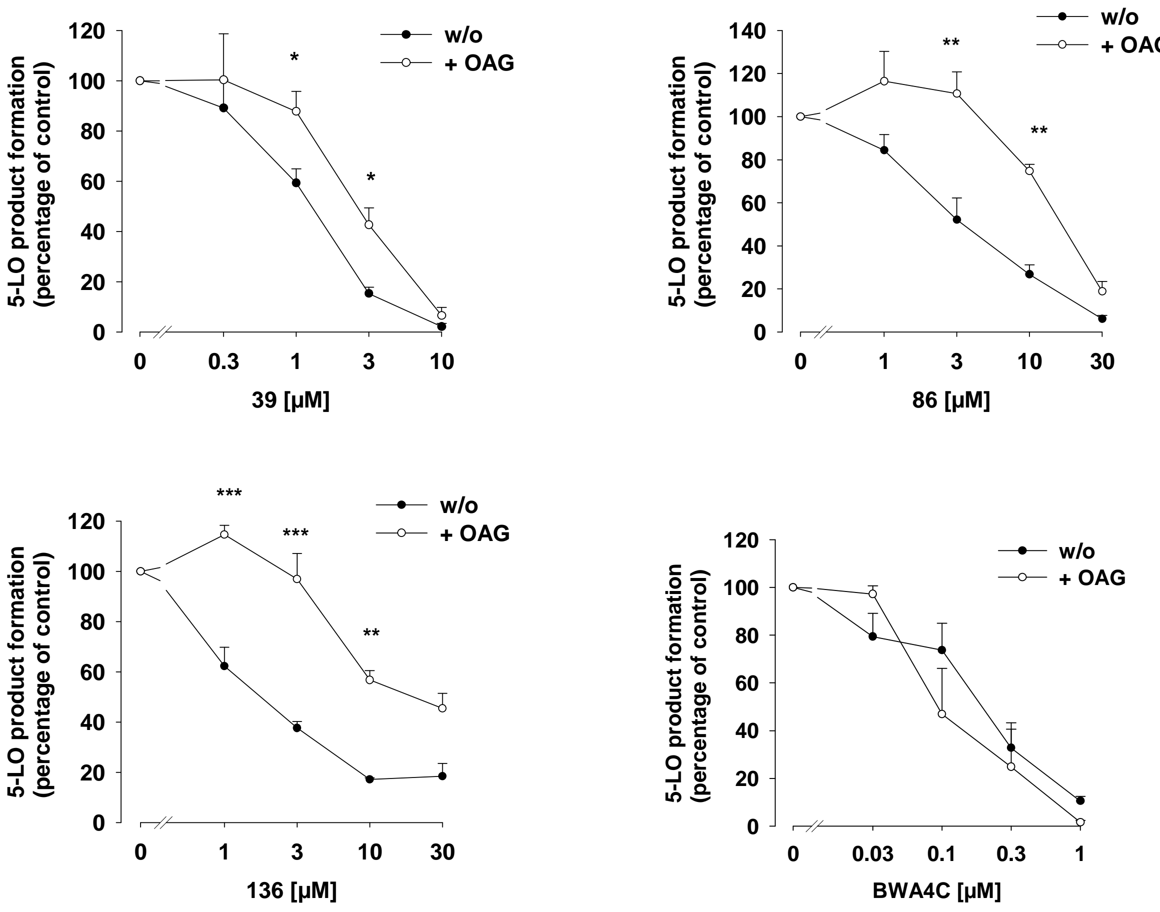


Fig. 33. Effects of OAG on 5-LO inhibition by the compounds. Partially purified human recombinant 5-LO (0.5 µg) expressed in *E.coli* was supplemented with 1 mM ATP and pre-incubated in 1 ml PBS buffer plus 1 mM EDTA with the compounds at the indicated concentrations or with vehicle (DMSO) for 5–10 min at 4 °C with or without 30 µM OAG as given in the figure. Samples were pre-warmed at 37 °C for 30 sec and 5-LO product formation was started by addition of 2 mM $CaCl_2$ plus 20 µM AA. After another 10 min, 5-LO product formation was determined. Values are given as mean + S.E., $n \geq 5$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. samples without OAG (w/o) at corresponding concentrations of inhibitors, ANOVA + Tukey post hoc test.

It has to be mentioned at this point that a different pattern was obtained if OAG (30 µM) was added prior to stimulation of PMNL with ionophore A23187. While the potency of **25** and **39** was impaired upon supplementation of OAG (**Fig. 34**), the inhibitory effect of **86** and **136** was hardly altered, and contrary to the expectations, the IC_{50} of BWA4C clearly shifted to higher values in presence of OAG in PMNL.

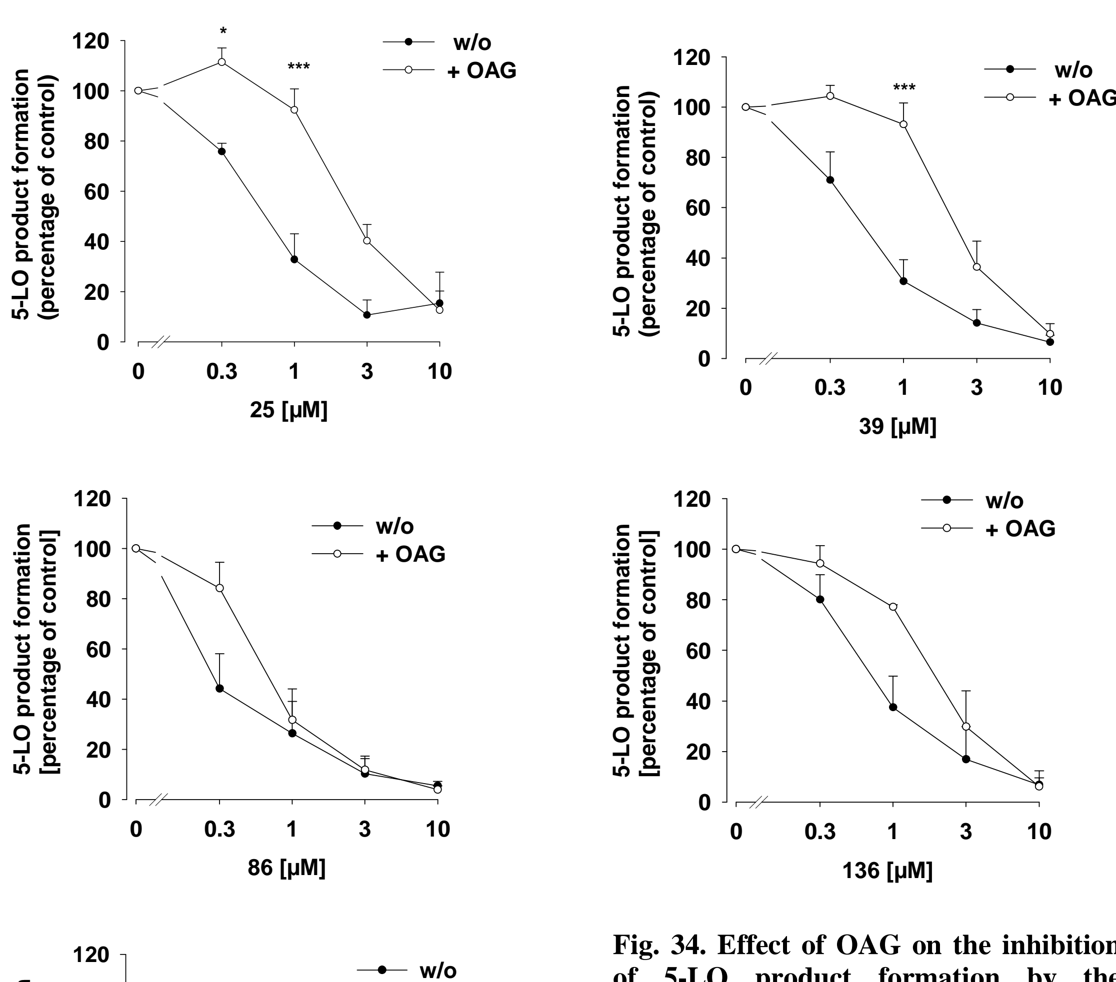


Fig. 34. Effect of OAG on the inhibition of 5-LO product formation by the compounds in PMNL. PMNL (5×10^6) were pre-incubated with test compounds for 15 min at 37 °C. OAG (30 µM) was supplemented 3 min before stimulation with 2.5 µM ionophore A23187 and 20 µM AA. The reaction was stopped after 10 min to analyze 5-LO product formation. Values are given as means + S.E., n = 3; *$p < 0.05$, ***$p < 0.001$ vs. samples without OAG (w/o) at corresponding concentrations of inhibitors, ANOVA + Tukey post hoc test.

The interaction between 5-LO and CLP is mediated by three tryptophanes (W13, W75 and W102) within the C2-like domain of 5-LO [108]. Recently, it was demonstrated in a pull-down assay that hyperforin is able to interrupt binding of 5-LO to CLP [210]. In order to evaluate if PA derivatives or 2-(phenylthio)hexanoic acid derivatives are also able to disturb the interaction between 5-LO and CLP, a fusion protein of CLP and glutathione-S-transferase (GST-CLP) was immobilized on GSH-sepharose beads, and CLP-bound 5-LO was determined by Western Blot. As expected, 5-LO bound to GST-CLP but not to GST alone (**Fig. 35a**). In one experiment, inclusion of **25** concentration-dependently decreased the amount of precipitated 5-LO, suggesting that **25** prevents binding of 5-LO to CLP. Unfortunately, the inhibitory effect of **25** could not be confirmed in subsequent experiments (**Fig. 35b**) and neither variation in the amount of 5-LO, GST-CLP, GSH-sepharose beads or BSA nor changes in buffer composition, reaction time or washing steps yielded consistent results (not shown). Compound **39** wasn't able to prevent the interaction between 5-LO and CLP either (**Fig. 35c**) and notably, also the expected effect of hyperforin could not be confirmed under the applied conditions.

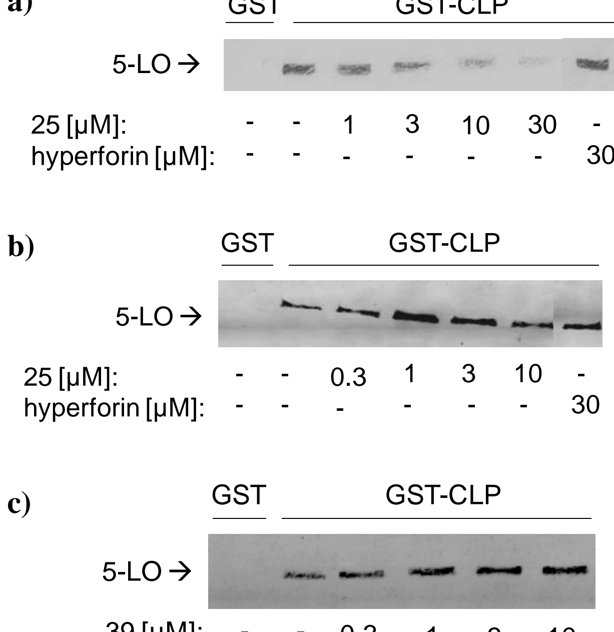


Fig. 35. Effect of 25 and 39 on the interaction between 5-LO and CLP. Purified human recombinant 5-LO (4 µg) was incubated with 40 µg of either GST (neg. control) or GST-CLP fusion protein linked to GSH-sepharose 4B beads in presence of BSA (50 µg/ml) and **25**, **39** or hyperforin, as indicated. After 30 min rotation at RT, washing and elution of the proteins with the same volume of SDS loading buffer, 5-LO was assayed by SDS-PAGE and Western Blot.

5.2.3. Investigation of a direct binding between PA derivatives and 5-LO

In order to confirm a direct binding of PA derivatives to 5-LO, surface plasmon resonance (SPR) spectroscopy studies were applied. The SPR method allows to monitor molecular interactions between an immobilized reactant (e.g. an enzyme) and a soluble component (e.g. a potential drug candidate) in real-time without the need to label the analytes [263], [264]. Thus, complex formation between two binding partners (and also their dissociation) implicates changes in the refractive index of the solvent near the surface that can be measured by the SPR biosensor. The binding responses are monitored in a sensogram as resonance units (RU).

A fusion protein of maltose-binding protein and 5-LO (MBP-5-LO, kindly provided by Prof. Dr. D. Steinhilber, University of Frankfurt) was immobilized on one of the two flow cells of a CM5 sensor chip, whereas MBP alone was applied on the other flow cell. Compounds **22, 25**, **39** or **41** (at different concentrations between 0.1 and 20 µM) were diluted into assay buffer, sequentially injected over the chip surface, and the resonance of the MBP-5-LO flow cell compared to the MBP control flow cell was determined. Unfortunately, no consistent binding pattern was obtained for any of the PA derivatives (data not shown). Neither variation of commercial assay buffers (± additional detergent) nor changes in the temperature or the flow rate improved the quality of the recorded sensograms. Possibly, due to their amphiphilic properties, PA derivatives form aggregates or micelles and thus bind to immobilized 5-LO in an unspecific and superstoichiometric manner [265].

5.2.4. Inhibition of 5-LO product formation by the compounds in human whole blood

Investigation of 5-LO product formation in human whole blood may translate to the *in vivo* effectiveness of a given test compound. Therefore, whole blood assays with 30 µM ionophore A23187 as stimulus were performed. As shown in **Fig. 36**, none of the representatives for PA or 2-(phenylthio)hexanoic acid derivatives (in concentrations up to 30 µM) was able to reduce 5-LO product synthesis under these conditions. The control inhibitor MK886 in contrast, showed a clear inhibitory effect.

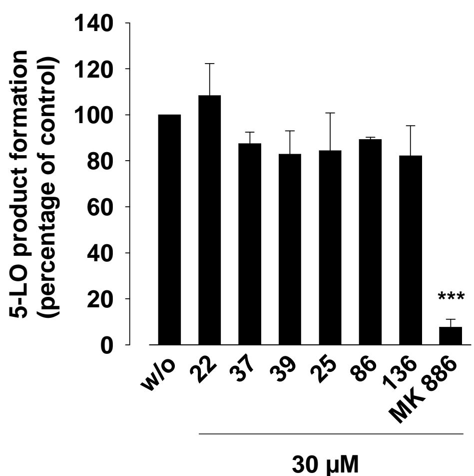


Fig. 36. Inhibition of 5-LO product formation in human whole blood. Freshly withdrawn blood (2 ml) was pre-incubated with compounds (30 µM, each) or vehicle (DMSO) for 15 min at 37 °C, and formation of 5-LO products was started by addition of 30 µM ionophore A23187 (10 min, 37 °C). The formation of 5-LO products was determined as described. Data are means + S.E.; $n \geq 3$; ***$P < 0.001$ vs vehicle (DMSO) control, ANOVA + Tukey post hoc test.

5.2.5. Investigation of the *in vivo* effectiveness of PA derivatives

5.2.5.1. Effects of PA derivatives on carrageenan-induced pleurisy in rats

Despite the lack of effectiveness in whole blood assays, one may not generally exclude *in vivo* efficacy of PA derivatives. For example, the compounds might enrich in inflammatory tissues and thus exert their anti-inflammatory actions. Therefore, **22** and **37**, two compounds out of an early synthesized set of PA derivatives that were inactive in human whole blood, were tested in carrageenan-induced pleurisy in rats, a well-established model of acute inflammation that involves 5-LO [266]. The experiments were performed by F. Dehm in the lab of Prof. Dr. L. Sautebin (University Naples, Italy). Injection of λ-carrageenan into the pleural cavity of rats provoked a massive accumulation of exudates containing inflammatory cells with elevated levels of LTB_4 but also of PGE_2 **(Fig. 37)**. Both **22** and **37** (1.5 mg/kg, given i.p., each, 30 min before carrageenan) significantly attenuated exudate formation and cell infiltration. Furthermore, the amounts of LTB_4 and PGE_2 in the exudates were clearly reduced by the compounds. The COX inhibitor indomethacin (5 mg/kg) also lowered these parameters except LTB_4 as expected.

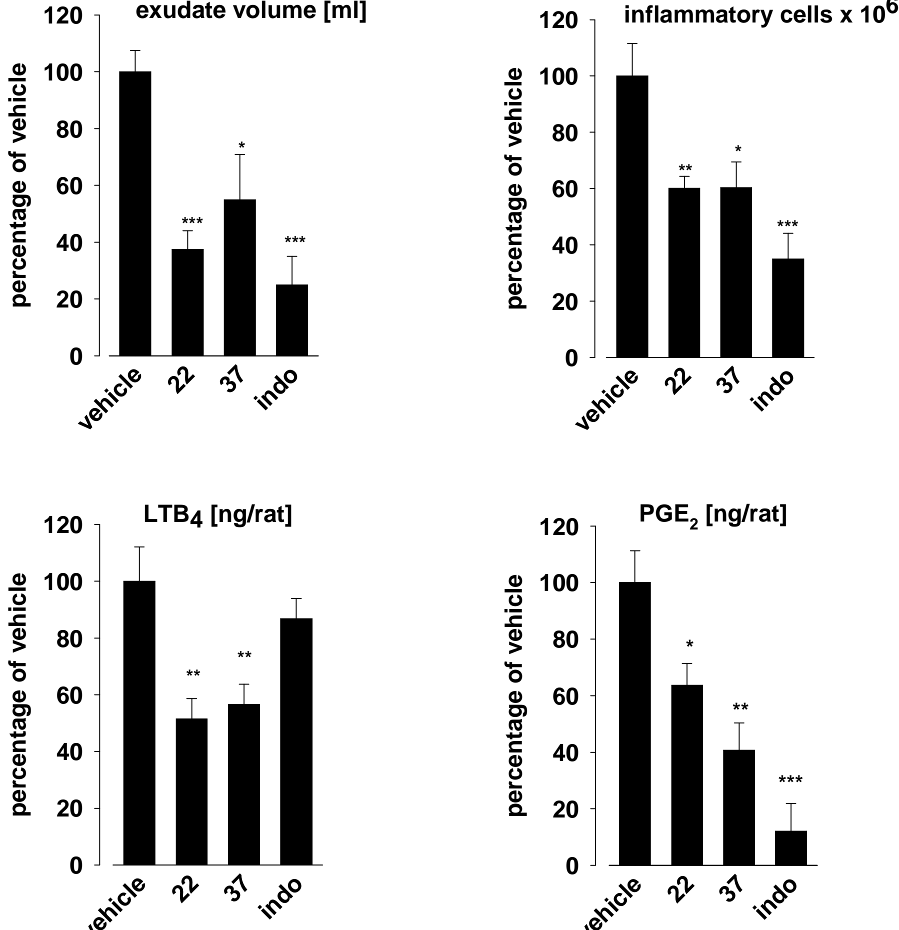


Fig. 37. Effect of 22 and 37 on carrageenan-induced pleurisy in rats. Thirty min before intrapleural injection of λ-carrageenan, rats were treated i.p. with **22**, **37** (1.5 mg/kg, each), indomethacin (5 mg/kg) or vehicle (DMSO). Exudate volume, inflammatory cell accumulation in the pleural cavity, LTB_4 and PGE_2 were assessed 4 h after λ-carrageenan injection. Data are means + S.E.; n = 7; *p<0.05, **p<0.01, ***p<0.01 vs. vehicle (4% DMSO). ANOVA + Tukey post hoc test. Performed by F. Dehm in the lab of Prof. Dr. L. Sautebin (University Naples, Italy).

5.2.5.2. Influence of compound 39 on PAF-induced shock

Encouraged by the promising results obtained for **22** and **37** in the pleurisy model, additional *in vivo* experiments with PA derivatives were performed by A. Rossi in the group of Prof. Dr. L. Sautebin. It was previously shown that 5-LO knockout mice are less sensitive to PAF-mediated lethal anaphylactic shock [267], [268], and that some 5-LO inhibitors as well as several FLAP inhibitors are able to prolong survival time in mice exposed to a lethal intravenous injection of PAF [269], [270, 271], [271]. To investigate whether or not PA derivatives also possess a protecting effect in this animal model, **39** was administered intraperitoneally 30 min prior to injection of a lethal dose of PAF (200 µg/kg). Indeed, 10 mg/kg of the compound prevented the mortality of the mice by almost 80% (**Table 5**). By comparison, 30 mg/kg of the FLAP inhibitor MK-886 have been shown in literature to increase survival to 50% [197].

compound	**survivors/total**
control	1/20 (1/5-0/5-0/5-0/5)
39 (1.5 mg/kg)	0/5
39 (5 mg/kg)	0/5
39 (10 mg/kg)	8/10 (5/5-3/5)

Table 5. Effect of 39 on PAF induced shock in mice. PAF was injected intravenously into female CD-1 mice at a dose of 200 µg/kg. **39** (1.5, 5, 10 mg/kg) or vehicle control (saline solution containing 2% DMSO) were administered intraperitoneally 30 min prior to PAF. The number of surviving animals after 1-2 h and the total number of animals tested for each group are given.

5.3. Further targets of PA derivatives and 2-(phenylthio)-hexanoic acid derivatives

5.3.1 Inhibition of mPGES-1, COX-1 and COX-2 by selected PA derivatives and 2-(phenylthio)-hexanoic acid derivatives

Simultaneous inhibition of both PG and LT synthesis is thought to be more efficient against inflammatory diseases than suppression of 5-LO products alone [211], [212], and efforts have been made over the past years to develop dual COX/5-LO inhibitors [272], [273]. Moreover, the dual inhibition concept was shown to be superior over single interference with COX enzymes in terms of gastrointestinal toxicity. However, COX-2 inhibition has been associated with an increased cardiovascular risk [216], and therefore, suppression of mPGES-1-derived PGE_2 formation in combination with 5-LO inhibition has been considered as an alternative to dual COX/5-LO inhibitors [18], [217]. In order to determine the effect of PA derivatives and 2-(phenylthio)hexanoic acid derivatives on mPGES-1, microsomal preparations of A549 cells were preincubated with selected compounds for 15 min, and then, PGH_2 was added to start PGE_2 formation. The assays were performed by M. Hieke, G. Kleefeld, A. Koeberle and D. Müller in our lab. The mPGES-1 inhibitor MK-886 was used as reference compound. As observed for 5-LO, PA itself was not able to block mPGES-1, but introduction of *n*-hexyl (**22**), *n*-octyl (**44**) or naphthyl (**63**) moieties in α-position of the carboxylic group yielded compounds that clearly reduced PGE_2 formation (**Table 6**). Replacement of the *o*-dimethylaniline moiety by a 6-aminoquinoline was not as beneficial as for 5-LO inhibition, and only the esterified compound **25** showed a remarkable reduction of PGE_2 (IC_{50} = 5.6 µM). Note that except **25**, all the other esters tested were rather poor inhibitors of mPGES-1. Introduction of bulky lipophilic residues at C6 of the pyrimidine, especially of biphenyl groups (**37**, **39**, **41**, **42**) also increased the potency of the free acids suggesting similar SARs for both 5-LO and mPGES-1.

Among the derivatives with bis-phenethoxy moieties, the most favorable profiles were displayed by trifluormethyl- (**94**, IC_{50} = 1.9 µM) and methyl- (**92**, IC_{50} = 0.9 µM) substituted compounds. Interestingly, replacement of the pyrimidine by phenyl and exchange of the two phenyl residues against bis-cyclohexyl yielded the highly efficient compound **113** (IC_{50} = 0.9 µM) that was less efficient in the 5-LO activity assay.

2-(Phenylthio)hexanoic acid derivatives turned out to potently inhibit mPGES-1 as well. Also for this class of compounds, voluminous lipophilic residues increased the efficacy with 3,4-

disubstituted (3-trifluoromethyl-4-chlorophenyl) **133** as the most effective representative (IC_{50} = 1.7 µM).

Taken together, a variety of PA derivatives and 2-(phenylthio)hexanoic acid derivatives act as dual 5-LO/mPGES-1 inhibitors that inhibit both enzymes about equally well. Due to the lipophilic acid character of these compounds, one could assume that they might also interfere with COX-1/2 by mimicking AA, the substrate of COX enzymes. Therefore, selected derivatives were assessed in cell-free assays using isolated ovine COX-1 and human recombinant COX-2, respectively. As shown in **Table 6**, most of the tested compound hardly blocked 12-HHT formation so that IC_{50} values below 10 µM were only reached for a few substances (COX-1: **30**, **32** and **63**; COX-2: **63** and **102**).

Compound	**mPGES-1** IC$_{50}$ or *remaining activity (± S.E.) at 10 µM*[d]	**COX-1** *remaining activity (± S.E.) at 10 µM*	**COX-2** *remaining activity (± S.E.) at 10 µM*
PA	n.i.[d]	n.d.	n.d.
7	*68.7 (± 4.0)*	n.d.	n.d.
8	*82.2 (± 3.4)*	n.d.	n.d.
13	*83.8 (± 7.4)*	n.d.	n.d.
14	n.i.	n.d.	n.d.
19	*70.0 (± 9.4)*	n.d.	n.d.
20	*79.9 (± 6.2)*	n.d.	n.d.
21	*80.7 (± 8.0)*	n.d.	n.d.
22	3.9	*75.2 (± 8.9)*[c]	*62.0 (± 5.6)*[c]
23	n.i.	n.d.	n.d.
24	*59.0 (± 6.5)*	n.d.	n.d.
25	5.6	*69.5 (± 1.9)*[c]	*61.7 (± 9.3)*[c]
26	*67.4 (± 7.3)*	n.d.	n.d.
28	*85.7 (± 8.9)*	n.d.	n.d.
30	2.6	*40.0 (± 8.9)*[c]	*70.4 (± 9.3)*[c]
32	n.d.	*45.1 (± 8.2)*	*57.6 (± 6.6)*
37	1.3	*67.3 (± 1.2)*[c]	*61.3 (± 3.7)*[c]
38	n.i.	n.d.	n.d.
39	1.6	*52.3 (± 0.9)*[c]	*57.1 (± 5.1)*[c]
41	1.7	*66.0 (± 8.3)*[c]	*61.2 (± 3.6)*[c]
41a	*36.8 (± 4.7)*	n.d.	n.d.
41b	*23.0 (± 5.9)*	n.d.	n.d.
42	2.1	*71.2 (± 1.0)*[c]	*53.7 (± 3.8)*[c]
44	*29.8 (± 7.1)*	n.d.	n.d.

Compound	**mPGES-1** IC$_{50}$ or *remaining activity (± S.E.) at 10 µM*[d]	**COX-1** *remaining activity (± S.E.) at 10 µM*	**COX-2** *remaining activity (± S.E.) at 10 µM*
45	*74.6 % (± 1.9)*	n.d.	n.d.
63	5.1	*42.2 % (±11.4)*[c]	*32.5 % (± 7.3)*[c]
83	*66.4 % (± 2.4)*	*91.8 % (± 2.8)*	*91.7 % (± 7.8)*
84	1.2	*72.2 % (± 7.7)*[c]	*86.0 % (± 12.6)*[c]
85	7.3	*84.6 % (± 3.2)*[c]	*56.9 % (± 0.8)*[c]
86	2.0	*61.8 % (± 9.8)*[c]	*63.8 % (± 9.7)*[c]
87	1.2	*64.2 % (± 8.8)*	*90.9 % (± 10.2)*
88	n.i.	n.d.	n.d.
89	3.6	*88.9 % (± 12.3)*[c]	*61.6 % (± 1.4)*[c]
90	2.2	*78.8 % (± 8.6)*[c]	*51.9 % (± 6.5)*[c]
91	0.9	*78.8 % (± 8.1)*[c]	*53.9 % (± 8.2)*[c]
92	0.9	*57.2 % (± 7.3)*	*93.3 % (± 11.6)*
93	*63.7 % (± 7.4)*	*72.8 % (± 10.3)*[c]	*94.6 % (± 13.5)*[c]
94	1.9	*54.4 % (± 10.6)*[c]	*69.0 % (± 12.4)*[c]
95	*88.3 % (± 6.3)*	*73.0 % (± 6.7)*	*75.8 % (± 5.0)*
96	3.7	*82.1 % (± 7.0)*	*91.6 % (± 8.4)*
97	5.9	*88.8 % (± 9.6)*	*82.6 % (± 14.1)*
98	6.5	*76.6 % (± 4.0)*	n.i.
99	2.7	*72.4 % (± 11.8)*[c]	*80.5 % (± 11.4)*[c]
100	9.9	*97.7 % (± 8.7)*	*87.9 % (± 3.4)*
101	*90.0 % (± 11.4)*	*68.2 % (± 4.0)*	*84.7 % (± 2.0)*
102	2.4	*52.7 % (± 6.4)*[c]	*49.5 % (± 10.7)*[c]
103	*62.4 % (± 8.4)*	*56.1 % (± 5.1)*	*50.1 % (± 2.1)*
104	4.6	*71.3 % (± 2.1)*	n.i.
105	3.4	*71.6 % (± 3.7)*	*92.5 % (± 2.7)*
106	2.6	*78.7 % (± 12.9)*[c]	*78.4 % (± 11.9)*[c]

Compound	**mPGES-1** IC$_{50}$ or *remaining activity (± S.E.) at 10 µM*[d]	**COX-1** *remaining activity (± S.E.) at 10 µM*	**COX-2** *remaining activity (± S.E.) at 10 µM*
107	3.1	*95.8 % (± 21.7)*[c]	*86.4 % (± 11.8)*[c]
108	5.0	*66.1 % (± 9.8)*[c]	*89.6 % (± 13.4)*[c]
109	*90.1 % (± 8.3)*	*99.1 % (± 12.1)*	n.i.
110	2.4	*63.8 % (± 16.6)*[c]	*66.5 % (± 11.0)*[c]
111	2.5	*69.1 % (± 4.0)*	*91.5 % (± 3.3)*
112	5.0	*82.0 % (± 6.7)*	*96.6 % (± 9.5)*
113	0.9	*73.8 % (± 8.7)*	*79.1 % (± 6.6)*
114	2.8	*81.4 % (± 8.5)*	*81.4 % (± 8.9)*
115	1.4	*68.0 % (± 12.1)*	*94.4 % (± 10.7)*
116	1.2	*86.8 % (± 24.3)*	n.i.
117	3.6	*76.4 % (± 7.3)*	n.i.
118	4.4	*81.0 % (± 11.8)*	n.i.
119	*55.1 % (± 5.4)*	*82.6 % (± 4.7)*	n.i.
120	*74.0 % (± 9.4)*	*92.3 % (± 9.2)*	n.i.
121	*81.9 % (± 5.6)*	n.d.	n.d.
122	2.9	n.i.[c]	n.i.[c]
122a	*19.6 % (± 8.9)*	n.d.	n.d.
122b	*17.0 % (± 8.0)*	n.d.	n.d.
123	*75.4 % (± 10.9)*	n.d.	n.d.
124	*41.1 % (± 3.9)*	n.d.	n.d.
125	*58.0 % (± 5.5)*	n.d.	n.d.
126	*42.6 % (± 2.7)*	n.d.	n.d.
127	*88.3 % (± 3.0)*	n.d.	n.d.
128	*59.1 % (± 6.5)*	n.d.	n.d.
129	*46.3 % (± 2.3)*	n.d.	n.d.

Compound	**mPGES-1** IC$_{50}$ or *remaining activity (± S.E.) at 10 µM*[d]	**COX-1** *remaining activity (± S.E.) at 10 µM*	**COX-2** *remaining activity (± S.E.) at 10 µM*
130	4.1	n.d.	n.d.
131	2.7	n.d.	n.d.
132	2.0	n.d.	n.d.
133	1.7	*71.3 % (± 2.0)*	n.i.
134	2.2	n.i.[c]	n.i.[c]
136	2.2	79.2[c]	n.i.[c]
138	n.i.	n.d.	n.d.
139	*83.9 % (± 3.6)*	n.d.	n.d.
140	*83.9 % (± 14.2)*	n.d.	n.d.
141	*26.3 % (± 10.0)*	n.d.	n.d.
142	*71.7 % (± 10.3)*	n.d.	n.d.
MK 886	2.1	n.d.	n.d.
indo-methacin	n.d.	*26.6 % (± 4.8)*	n.d.
celecoxib	n.d.	n.d.	*34.3 % (± 10.4)*

Table 6. Inhibition of cell-free mPGES-1, isolated ovine COX-1 and human recombinant COX-2. Data are means ± S.E.; n ≥ 3

[c]experiments were performed by G. Kleefeld or D. Müller in the lab of Prof. Dr. O. Werz (University of Tuebingen, Germany).

[d]experiments were performed by M. Hieke, G. Kleefeld, Dr. A. Koeberle or D. Müller in the lab of Prof. Dr. O. Werz (University of Tuebingen, Germany).

5.3.2. Short term effects of selected PA derivatives on typical neutrophil functions

Activation of PMNL by the pro-inflammatory fMLP does not only activate 5-LO but also induces transduction pathways that mediate adhesion, degranulation, chemotaxis and generation of reactive oxygen species (ROS) in order to counteract bacterial infection or tissue damage [274]. fMLP binds to G-protein-coupled receptors (namely FPR and FPRL1) on the cell surface, and subsequent activation of PLC, PLD, PLA_2, phosphatidylinositol-3-kinase (PI3K) and tyrosine phosphorylation transduces the signal to the respective second messengers [275]. Various kinases including protein kinase C (PKC) and MAPKs become activated and mediate distinct cellular effects such as Ca^{2+} mobilization, ROS formation and release of human leukocyte elastase (HLE).

The effects of PA and selected quinolinic derivates on typical immediate neutrophil functions have previously been investigated in our group. Thus, it was demonstrated that derivatives with α-butyl or α-hexyl substitution (in particular the free acids) but not PA itself or the α-unsubstituted quinolinic derivative attenuate ROS formation and inhibit HLE secretion as well as intracellular Ca^{2+} mobilization [245].

Many neutrophil functions are mediated by the activation of p38 MAPK and ERKs, and in particular p38 MAPK might be a target for the treatment of inflammatory diseases [276]. In order to test if the suppression of ROS, HLE and Ca^{2+} is the result of an interference of the compounds with MAPK, PMNL were preincubated with the PA derivatives for 15 min, and phosphorylation (and thus activation) of p38 MAPK and ERK-1/2 was induced with fMLP. As **Fig. 38A** shows, the α-butyl- (**16**) and α-hexyl-substituted (**24**) acidic derivatives clearly reduced phosphorylation of p38 MAPK but not of ERK-2. In accordance with the preceding results, neither PA nor the unsubstituted **2** or the esterified **25** affected the phosphorylation of these kinases. Since **39**, **86** and **136** were ineffective as well (**Fig. 38B**), it is assumed that the effect on p38 MAPK is exclusive for quinolinic PA derivatives.

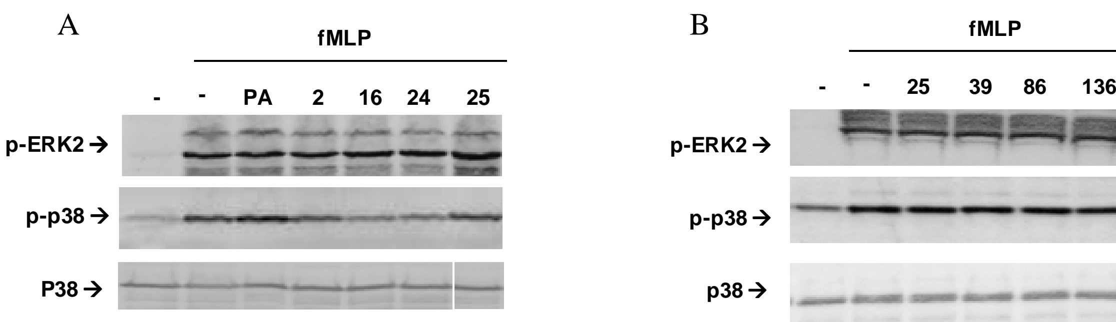


Fig. 38. Influence of the compounds on MAPK. PMNL were resuspended in PGC buffer (10^7/100 µl) and pre-incubated with the test compounds (10 µM, each) or vehicle (0.3% DMSO) for 15 min at 37 °C. Then, 1 µM fMLP was added, and the incubation was continued for 1.5 min. The reaction was stopped by addition of 100 µl SDS loading buffer, and samples were analyzed for phosphorylated p38 MAPK (p-p38 MAPK) and phosphorylated ERK-2 (p-ERK2) by SDS-PAGE and Western Blot. Ponceau S-staining of the membranes (not shown) and analysis of total p38 MAPK protein assured equal protein loading in each lane. Similar results were obtained in three additional independent experiments.

5.3.3. Discovery of additional targets of PA derivatives and 2-(phenylthio)hexanoic acid derivatives by a fishing approach

One classical approach to identify the targets of a certain compound is to investigate systematically its effect on distinct sections of signaling networks in order to find out the interaction partner. A more recent and time saving method is to perform selective protein fishing experiments by immobilizing the compound of interest to an insoluble biocompatible resin. This construct is incubated with a cell lysate as target source and subsequently centrifuged in order to pull down proteins that bind specifically to the immobilized molecule. The fished proteins can be separated by SDS-PAGE, and those which bound exclusively to the resin with the compound of interest (but not to a negative control) are selected, cut out and identified by MALDI or ESI mass spectrometry.

5.3.3.1. Target fishing with a quinolinic PA derivative

Considering the multiple effects of quinolinic PA derivatives on neutrophil functions, it was interesting to gain more insight into the exact target(s) of the compounds. Possibly, the inhibitory effect on intracellular Ca^{2+} levels and p38 MAPK phosphorylation is mediated by an interference of the derivatives with signaling cascades connecting fMLP stimulation with p38 MAPK and Ca^{2+} mobilization. In order to elucidate potential targets of PA derivatives, protein fishing experiments were performed with **24**, a compound that potently inhibits LTB_4 formation, ROS production, HLE release, Ca^{2+} mobilization and p38 MAPK phosphorylation in PMNL. The compound was coupled via its acidic group to an amino-functionalized methacrylic acid resin (**Fig. 39A**) and incubated with freshly prepared PMNL lysates. Gel electrophoresis followed by silver staining of the fished proteins revealed a protein with an apparent mass of ~ 25 kDa as putative candidate (**Fig. 40A**) that could neither be detected in control samples where beads without ligand were used nor in a sample with immobilized PA (structure: **Fig. 39B**). MALDI mass spectrometric and subsequent data base analysis identified the candidate protein as human NRH:quinone oxidoreductase 2 (NQO2, also referred to as QR2), and a direct interaction with **24** was confirmed by incubation of **24**-beads with isolated recombinant NQO2 followed by SDS-PAGE and Western Blot with NQO2 antibody (**Fig. 40B**).

Fig. 39. Structures of immobilized 24 (A) and PA (B). 100 µmol of **24** or PA, respectively, were solubilized in 500 µl DMF and mixed with 500 µl pre-washed amino-functionalized methacrylic acid resin (Toyopearl AF-Amino-650M, Tosho Bioscience). After addition of 65 µl DIC, samples were rotated for 48 h at pH 4.5 and RT. Remaining free amino groups of the matrix were blocked with 10% acetic acid and 100 µl DIC for 12 h. Then, the beads were washed several times with water and stored in 20% (v/v) ethanol at 4 °C.

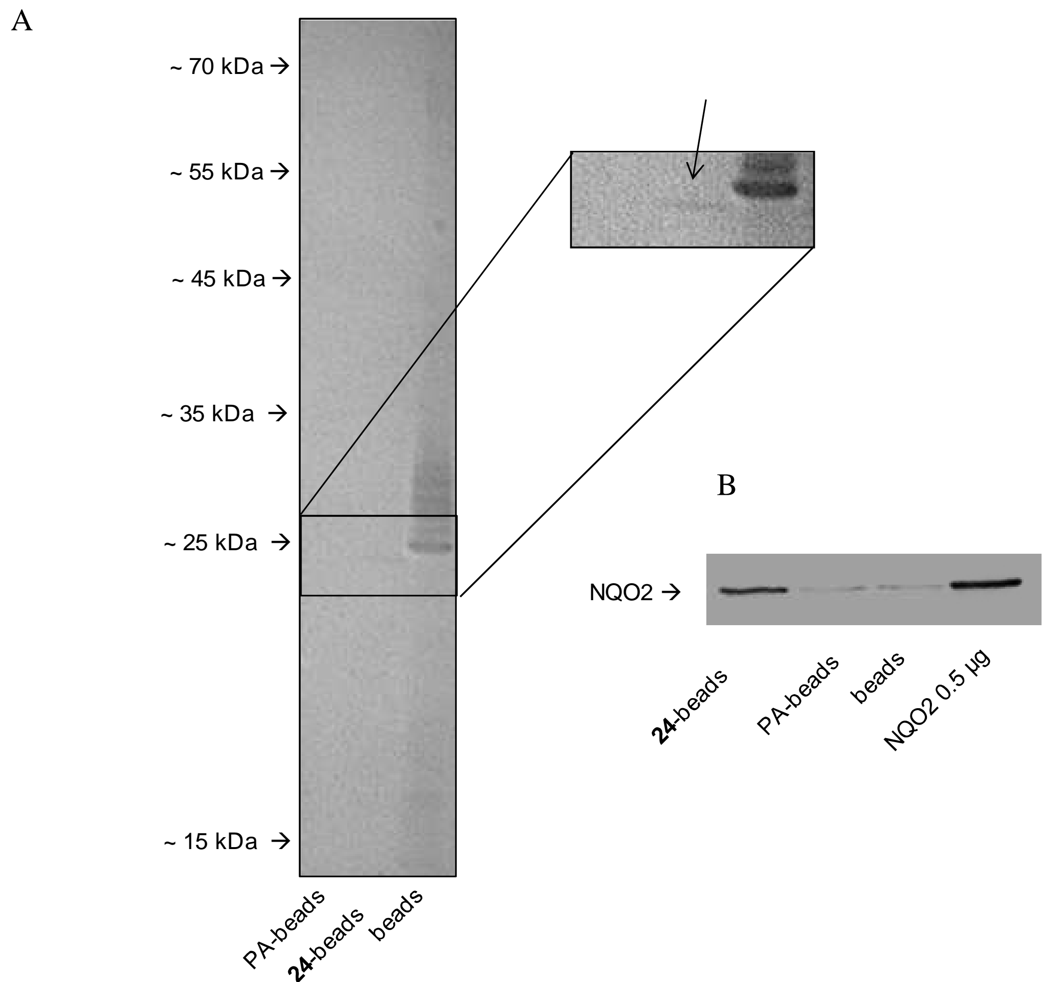


Fig. 40. Protein fishing with beads carrying 24 ("24-beads"). (A) 50 µl prewashed "**24**-beads", PA-beads or pure beads, respectively, were incubated with cell lysate from 10^8 freshly prepared PMNL in 700 µl binding buffer + 1% Triton X-100 for 12 h at 4 °C under rotation. Beads were washed three times with binding buffer + 1% Triton X-100, boiled with 2× SDS loading buffer for 5 min at 96 °C and SDS-PAGE followed by silver staining was performed. The band of interest (at ~ 25 kDa) was cut out, digested and peptides were analyzed by MALDI mass spectrometry. Similar results were obtained in two independent experiments. **(B)** 50 µl prewashed **24**-beads, PA-beads or pure beads, respectively, were incubated with 0.5 µg human recombinant NQO2 in 500 µl binding buffer + 1% Triton X-100 in presence of 0.1 µg/ml BSA for 12 h at 4 °C under rotation. Beads were washed three times with binding buffer + 1% Triton X-100, boiled with 2× SDS loading buffer for 5 min at 96 °C, and a Western Blot was performed. Similar results were obtained in three independent experiments.

NQO2 activity assay

Protein fishing experiments give information about the physical interaction of two binding partners, but the functional consequences of this interaction remains to be elucidated in appropriate *in vitro* or *in vivo* assays.

Human NQO2 is a 26 kDa, FAD-dependent flavoenzyme that catalyzes two-electron reductions of quinones to hydroquinones. This reaction counteracts one-electron reductions of quinones that lead to the formation of ROS [277]. If the detoxifying properties of NAD(P)H:quinone oxidoreductase (NQO1) are well characterized, the role of NQO2 is not fully understood yet [278]. The hypothesis that NQO2 is also involved in detoxification processes is supported by the fact that the enzyme is thought to reduce carcinogenic estrogen ortho-quinones [279] and that NQO2-/- mice are more susceptible to polycyclic aromatic hydrocarbon-induced skin carcinogenesis [280]. In addition, some anti-malarian drugs (quinolines) inhibit NQO2 and this may cause red blood cell oxidative stress leading to the death of the plasmodia [281]. However, other studies revealed that inhibition of NQO2 by resveratrol renders cells less sensitive to toxic menadione [282], and NQO2-/- mice showed a higher tolerance towards menadione [283]. Moreover, it was demonstrated that NQO2 is identical with the melatonin binding site MT3 so that there might be a relation between NQO2 inhibition by melatonin and its antioxidant effects [284]. Another group reported reduced proliferation rates upon knock-down of NQO2 in a chronic myeloic leukemia (CML) cell line [285]. Taken together, some data indicate that NQO2 might also be responsible for the transformation of quinone substrates into more reactive compounds that cause cellular damage and thus, inhibition of NQO2 could be beneficial in pathophysiological situations associated with oxidative stress. Therefore, it was interesting to investigate whether binding of quinolinic PA derivatives to NQO2 is associated with inhibition of the enzyme´s activity and what consequences would result from such interference. The ability of pure recombinant NQO2 (10 ng) to reduce menadione (100 µM) in presence of **24** or **25**, respectively, was measured by UV-Vis spectroscopy with colororimetric MTT as a reporter dye. As **Fig. 41A** shows, **24** (10 µM) but not its esterified analog **25** slightly attenuated the activity of NQO2 after 5 min reaction time. In contrast, the control inhibitor resveratrol (10 µM) exhibited a very potent reduction of the formazan product that reflects the amount of enzymatically converted menadione. As expected, PA itself had no influence on the enzyme reaction.

In order to check if the inhibitory effect of **24** on NQO2 activity increases with longer incubation times, a time course of the enzymatic reaction was performed. However, also after 15 min, no increased potency of **24** could be observed (**Fig. 41B**).

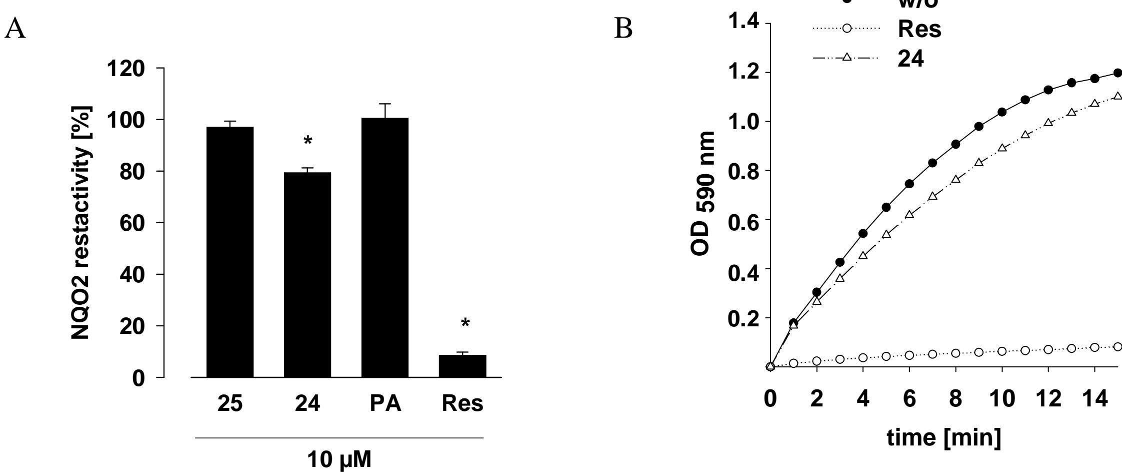


Fig. 41. NQO2 activity assay. (A) Human recombinant NQO2 (10 ng) was pre-treated with test compounds, resveratrol (10 µM each) or vehicle in 200 µl assay buffer including the cofactors FAD (1 µM) and SCHP (0.5 mM) for 5 min at RT. The enzyme reaction was started with menadione (100 µM) plus MTT (0.134 mg/ml), and after 5 min, the absorbance was measured at 590 nm. Data are means + S.E. n = 3. **(B)** Human recombinant NQO2 (10 ng) was pre-treated with **24**, resveratrol (10 µM each) or vehicle in 200 µl assay buffer including the cofactors FAD (1 µM) and SCHP (0.5 mM) for 5 min at RT. The enzyme reaction was started with menadione (100 µM) plus MTT (0.134 mg/ml) and the absorbance (at 590 nm) was measured at the indicated time points. Similar results were obtained in three independent experiments.

5.3.3.2. Target fishing with a biphenylic PA derivative

As described above, biphenylic PA derivatives are among the most potent compounds tested in this study regarding 5-LO and mPEGS-1 inhibition. To investigate whether they also interfere with other targets in PMNL, fishing experiments were performed with a biphenylic compound derived from **39**. Since the acidic group is essential for the inhibitory effect on both 5-LO and mPGES-1, this moiety was not altered. Instead, compound **143** possessing a carboxylic group in para position of the biphenylic residue of **40** (the ethylester of **39**) was synthesized by M. Dittrich

in the lab of Prof. Dr. M. Schubert-Zsilavecz and immobilized to an amino-functionalized methacrylic acid resin via the free carboxylic group. To obtain the designated fishing construct (**Fig. 42A**), the ethylester was cleaved with LiOH. The corresponding α-unsubstituted compound **144** was synthesized accordingly and used as a control (**Fig. 42B**). Incubation of the immobilized compounds with PMNL lysate was performed as described above, but in this case, proteins were eluted with 4 M urea pH 8.5, instead of SDS. Proteins fished with **143** were labeled with the fluorescence dye Cy5, whereas the eluate of the **144** sample was labeled with Cy3. Both eluates were mixed and proteins were separated by IEF in the first dimension followed by SDS-PAGE in the second dimension. The gel was scanned with an ETTAN DIGE Imager (GE Healthcare). As **Fig. 43A** shows, four spots were clearly more intensive in the channel for Cy5 (red) versus the channel for Cy3 (green). Unfortunately, the protein amount of three of these spots was under the detection limit for silver staining (**Fig. 43B**) so that they could not be picked for MS analysis. Spot number 1 was analyzed by MALDI MS, but turned out to be an unspecific impurity.

A

H N N S O OH N H N beads Cl O

B

H N N S O OH N H N beads Cl O

Fig. 42. Structures of immobilized 143 (A) and 144 (B). 100 µmol of **143** or **144**, respectively, were solubilized in 500 µl DMF and mixed with 500 µl pre-washed amino-functionalized methacrylic acid resin (Toyopearl AF-Amino-650M, Tosho Bioscience). After addition of 65 µl DIC, samples were rotated for 48 h at pH 4.5 and RT. Remaining free amino groups of the matrix were blocked with 10% acetic acid and 100 µl DIC for 12 h. After several washing steps, the ester group of **143** and **144**, respectively, was cleaved with LiOH (300 µmol) in THF/H_2O (3:1). After 24 h rotation at RT, beads were washed and stored at 4 °C.

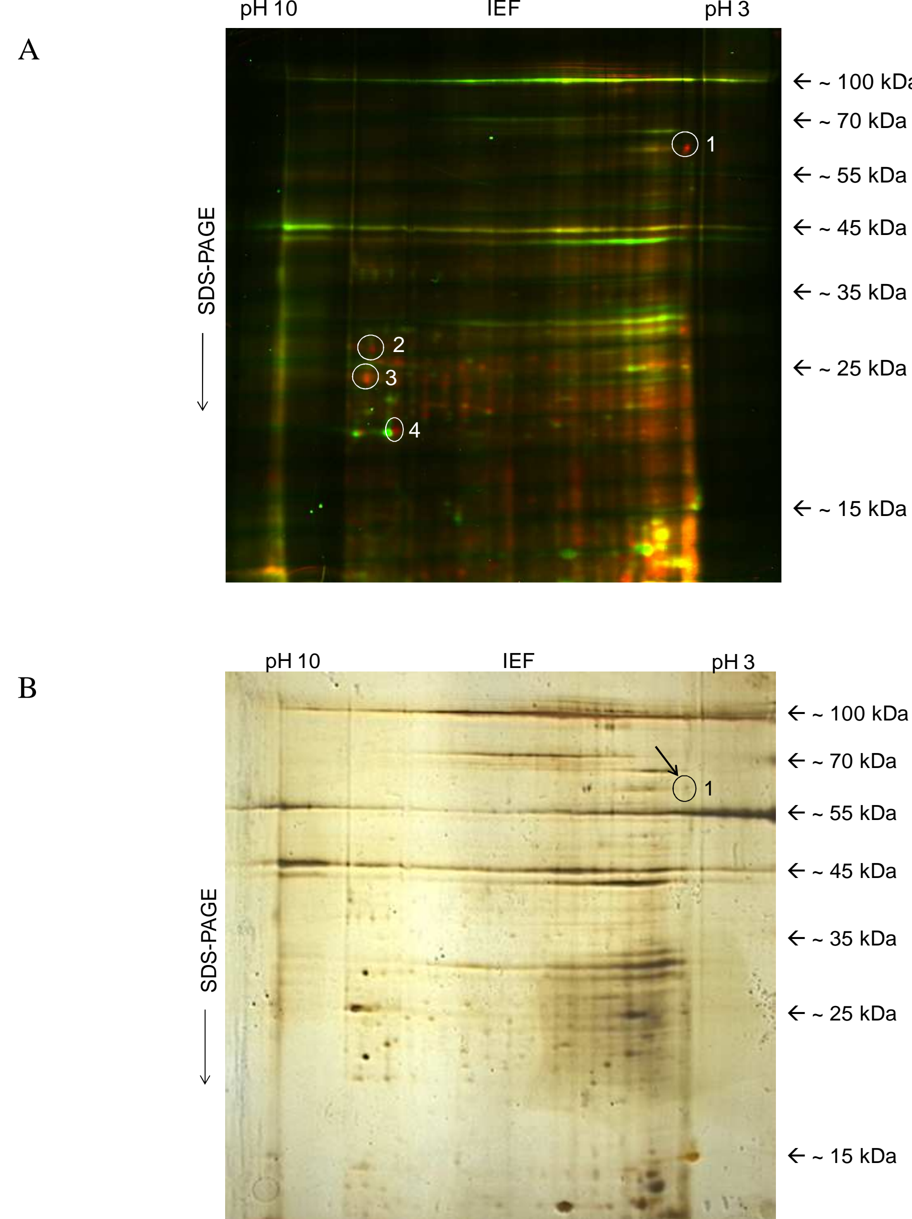


Fig. 43. 2D-DIGE of proteins fished with 143 from a PMNL cell lysate. **(A)** 50 µl pre-washed **143**-beads or **144**-beads, respectively, were incubated with cell lysate from 1.5 × 10^9 freshly isolated PMNL in 700 µl binding buffer + 0.1% Triton X-100 for 12 h at 4 °C under rotation. Beads were washed three times with binding buffer + 0.1% Triton X-100. Bound proteins were eluted with 4 M urea, pH 8.5, and the protein amount was determined. Aliquots of the fished proteins were labeled with Cy5 (for **143**) or Cy3 (for **144**). Samples were mixed, and proteins were separated by IEF in the first dimension followed by SDS-PAGE in the second dimension. The gel was scanned with an ETTAN DIGE Imager (GE Healthcare). Red: Cy5 labelled **143**-fished proteins, green: Cy3 labelled **144**-fished proteins (neg. control). **(B)** Silver staining of the 2-D-DIGE gel from (A). Spot number 1 was picked and analyzed by MALDI-MS.

In another fishing experiment with **143**, proteins were separated with one-dimensional SDS-PAGE. Several bands between 100 and 130 kDa were more intensive in the eluate of the **143**-beads compared to the bands obtained with "negative" control **144**-beads (although the total amount of fished proteins was clearly higher with **144**-beads), but the protein amount was too small to be cut out for MS analysis (**Fig. 44**).

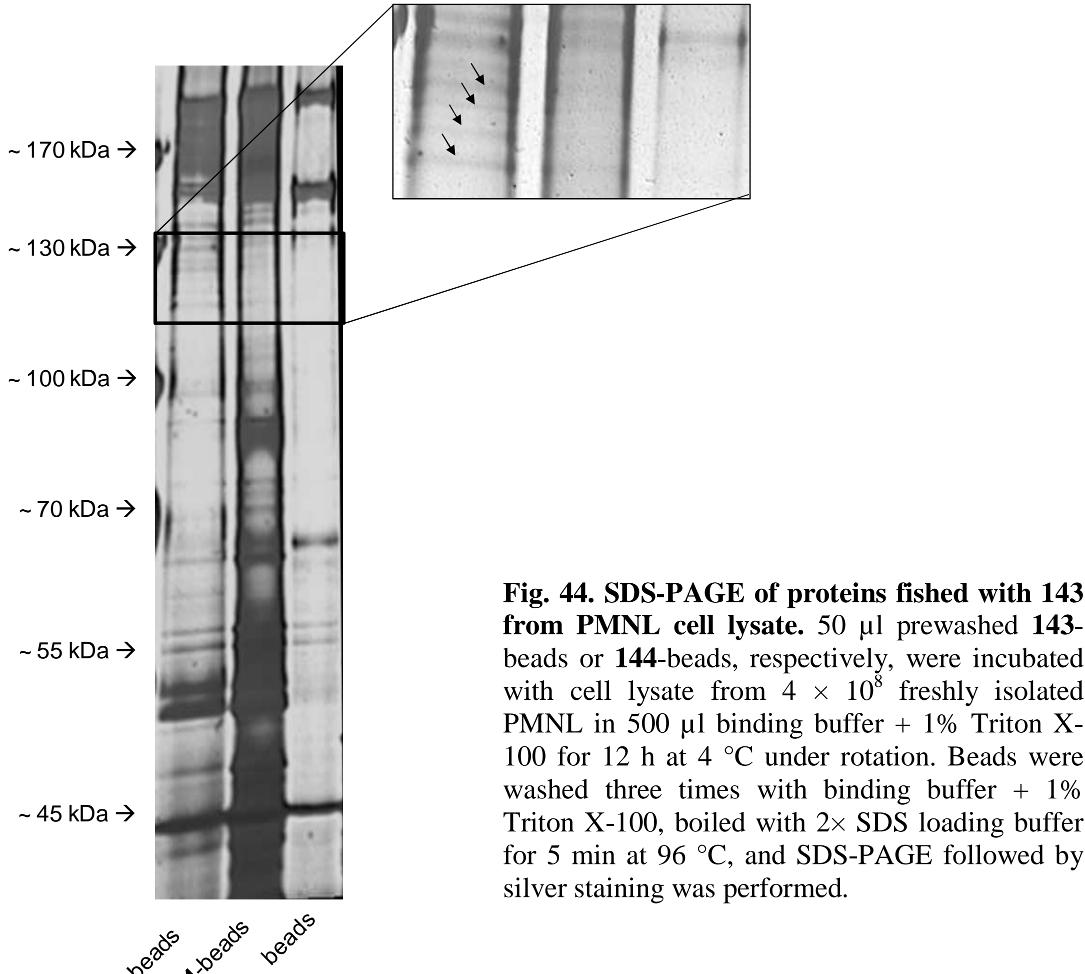


Fig. 44. SDS-PAGE of proteins fished with 143 from PMNL cell lysate. 50 µl prewashed **143**-beads or **144**-beads, respectively, were incubated with cell lysate from 4 × 10^8 freshly isolated PMNL in 500 µl binding buffer + 1% Triton X-100 for 12 h at 4 °C under rotation. Beads were washed three times with binding buffer + 1% Triton X-100, boiled with 2× SDS loading buffer for 5 min at 96 °C, and SDS-PAGE followed by silver staining was performed.

5.3.3.3. Target fishing with a 2-(phenylthio)hexanoic acid derivative

In order to identify additional binding partners of 2-(phenylthio)hexanoic acid derivatives, a compound resembling **122** was synthesized by M. Dittrich in the lab of Prof. Dr. M. Schubert-Zsilavecz. Again, the carboxylic acid was conserved so that an additional functional group was introduced at the *o*-dimethyl moiety of **122**. Because of synthetic reasons, an amino residue was inserted instead of a carboxylic group yielding compound **145**. Again, a compound lacking α-substitution was synthesized as "negative" control (**146**). Both molecules were immobilized via their amino group to an epoxy-activated matrix (Toyopearl AF-Epoxy 650M, Tosho Bioscience) (**Fig. 45**), and fishing experiments were performed with PMNL lysates. As **Fig. 46** shows, the band patterns for **145** and **146** were identical, and no additional bands could be detected compared to the blocked matrix alone.

In another fishing experiment with **145**, bound proteins were eluted with 4 M urea, and 2D-DIGE separation was performed. **145** bound proteins were labeled with Cy3, whereas proteins eluted from the negative control **146** were marked with Cy5. Unfortunately, almost no spots were detectable after scanning of the gel with the ETTAN DIGE Imager (not shown).

A

B

Fig. 45. Structures of immobilized 145 (A) and 146 (B). 40 µmol of **145** or **146**, respectively, were solubilized in 400 µl DMF and mixed with 75 mg pre-washed epoxy-activated resin (Toyopearl AF-Epoxy-650M, Tosho Bioscience). Samples were rotated for 72 h at pH 9-13, RT. Remaining free epoxy groups of the matrix were blocked with ethanolamine at pH 9-13 for 12 h. Beads were washed and stored in 20% (v/v) ethanol at 4 °C.

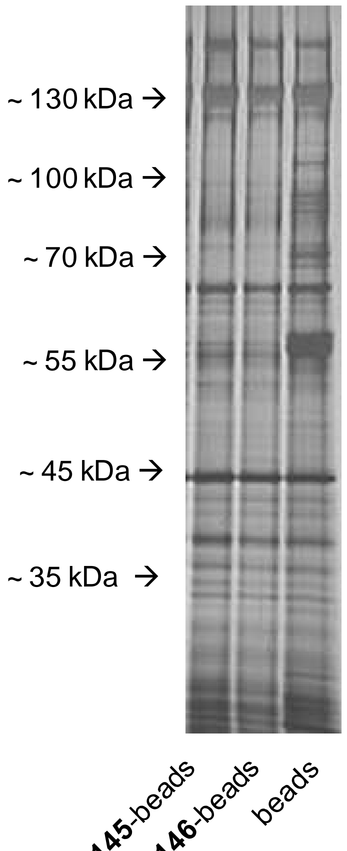


Fig. 46. Protein fishing with 145-beads. 50 µl prewashed **145**-beads or **146**-beads, respectively, were incubated with cell lysate from 4 × 10^8 freshly prepared PMNL in 500 µl binding buffer + 1% Triton X-100 for 12 h at 4 °C under rotation. Beads were washed three times with binding buffer + 1% Triton X-100, boiled with 2× SDS loading buffer for 5 min at 96 °C, and SDS-PAGE followed by silver staining was performed..

Finally, we attempted to confirm a direct interaction between 5-LO and PA derivatives or 2-(phenylthio)hexanoic acid derivatives, respectively, using a fishing experiment. However, it was not possible to selectively precipitate 5-LO from PMNL lysates nor from a preparation of partially purified enzyme with any of the immobilized derivatives described above (not shown).

6. Discussion

LTs are local acting messengers that play established roles in asthma and allergic rhinitis, but also seem to contribute to other inflammatory diseases, cancer and cardiovascular diseases [51], [191]. The first two steps in LT biosynthesis are catalyzed by the nonheme iron-containing 5-LO, which is therefore an interesting target for therapeutic intervention [66]. Although a lot of research effort has been made on 5-LO inhibitors during the past decades, most potential candidates were not effective in clinical studies or caused severe side effects [262]. However, regarding the growing number of studies that confirm key roles of LTs in major diseases, the development of selective and safe drugs that interfere with LT biosynthesis is a great challenge.

This study addresses the discovery of 5-LO inhibition by a large set of derivatives of PA and of 2-(phenylthio)hexanoic acid that were synthesized in the group of Prof. Dr. M. Schubert-Zsilavecz (University of Frankfurt). In addition, mechanistic aspects of selected compounds were investigated in various cell-based and cell-free assays so that a detailed analysis of the interference of these derivatives with the 5-LO enzyme is provided.

The second part of this work focuses on other anti-inflammatory targets of the compounds and describes protein fishing experiments that were performed in order to reveal novel targets of PA derivatives and 2-(phenylthio)hexanoic acid derivatives.

6.1. SARs of PA derivatives and 2-(phenylthio)hexanoic acid derivatives as 5-LO inhibitors

PA did not influence 5-LO product formation, but structural variations at different positions of the molecule led to a clear inhibitory effect. Thus, replacement of the *o*-dimethylaniline of PA by bulkier lipophilic residues (especially biphenyls) in combination with introduction of α-hexyl or α-naphtyl moieties yielded potent inhibitors of 5-LO product formation in PMNL with **72** as the most effective representative (IC_{50} = 0.1 µM). The compounds directly inhibited 5-LO although their potency was somehow decreased for the purified enzyme. Presumably, additional mechanisms contribute to the reduction of LT biosynthesis in intact cells. Alternatively, there might be some remaining cellular components from crude homogenates of *E. coli* present that attenuate the inhibitory effect of the compounds as observed in the 40,000×g (S40) supernatants from *E. coli* lysates (see below). A similar effect of cellular components was reported for 5-LO inhibition by hyperforin [210]. However, a clearly decreased IC_{50} value was obtained for the 2,3-

dihydro-indene derivative **7** in assays with partially purified 5-LO compared to PMNL (0.8 µM vs. 3.9 µM), a fact that cannot be readily explained.

Notably, only the free acids were able to suppress 5-LO products in cell-free assays, whereas the esterified compounds had no effect. However, in intact cells, the esters of PA and derivatives with α-alkyl chains up to butyl or with α-phenyl residues possessed a higher potency than their acidic analogs, whereas insertion of long and bulky alkyl/aryl groups abolished the need for esterification. Obviously, the lipophilic acid character (mimicking AA) is important for a direct interference with 5-LO, and inhibition of cellular LT formation by esters with rather small and less hydrophobic substituents might be mediated by interaction with different cellular mechanisms that influence LT biosynthesis. Alternatively, esters which are less bulky might be cleaved more easily by esterases. Interestingly, esters were also more effective in PMNL than their corresponding free acids upon insertion of a quinoline or a 3-pyridylethylamine residue, regardless of the α-substitution. Various inhibitors of LT formation possess quinolinic structures, but in general, these compounds are more potent inhibitors of FLAP than of 5-LO [286]. Due to the increased inhibitory efficacy in PMNL compared to the purified enzyme, one may speculate that the compounds influence both 5-LO and FLAP. However, in contrast to FLAP inhibitors [165], no gender difference could be shown in the potency of quinolinic PA derivatives and addition of exogenous AA did not impair the inhibitory effect of **25** obtained after stimulation of PMNL with ionophore A23187 alone.

PA derivatives with two phenylalkyloxy or alkyloxy residues at the pyrimidine core together with α-alkyl substituents turned out to be potent 5-LO inhibitors as well. Again, only the free acids were active, and a discrepancy between the IC_{50} values in PMNL versus cell-free assays could be observed. Insertion of *para*-substituents at the phenethoxy moieties, in particular methoxy-groups, improved the efficacy of the compounds. In cell-based assays, replacement of the two phenyl residues by bioisosteric thiophenes (**98**) or cyclohexyl groups (**102**) was possible without any loss of efficacy, whereas introduction of smaller or larger aliphatic residues was rather detrimental, indicating defined target interactions at this position. Interestingly, reduction or enlargement of the aliphatic ring size barely influenced the IC_{50} values in cell-free assays. Replacement of the pyrimidine core by a benzene group together with exchange of the sulfur against oxygen or an ethenyl group was tolerated, whereas the corresponding 3-phenyl-propionic acid derivatives were inactive, suggesting that a fixed steric configuration at this part of the

molecule is advantageous. Interestingly, the 2,5-diphenethoxy-substituted **110** was the only 3-phenyl-propionic acid derivative that was equally effective as the compounds with an ethenyl group, possibly due to steric reasons.

2-(Phenylthio)hexanoic acid derivatives combine the beneficial 2-mercapto-hexanoic acid part of PA derivatives with a phenolic backbone, etherified with a phenoxypropyl moiety. Similar SARs as for PA derivatives were observed for this class of compounds. Thus, α-alkyl substitution was important, and bulky lipophilic substituents at the phenoxypropyl moiety increased the inhibitory potency. The most effective inhibition of 5-LO product formation in PMNL was obtained by the biphenylic **136** (IC_{50} = 0.9 µM) and by the quinoline substituted ester **139** (IC_{50} = 0.8 µM). Note that upon introduction of the quinoline, the esterified form was superior over the free acid as observed for the corresponding PA derivatives.

Taken together, common structural features of the most effective compounds out of these SAR studies are i) bulky lipophilic substituents in α-position of the carboxylic acid, in particular *n*-hexyl or naphthyl groups, ii) an aromatic group in the center of the molecule (i.e. pyrimidine or benzene) and iii) one or (in case of the bis-phenylalkyloxy or bis-alkyloxy substituted derivatives) two residues forming a lipophilic backbone.

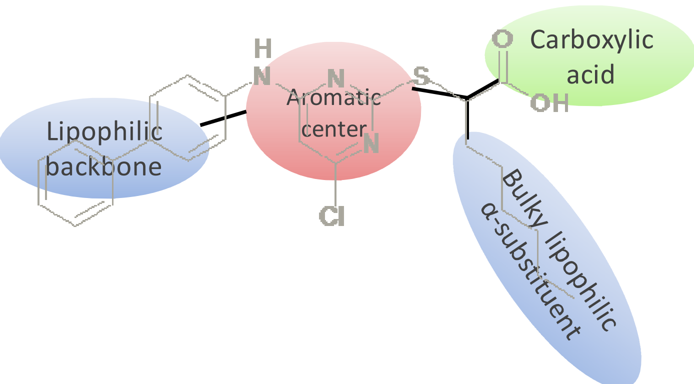


Fig. 47. Common structural features of PA derivatives and 2-(phenylthio)hexanoic acid derivatives with a potent inhibitory effect on 5-LO. The most effective 5-LO inhibition was reached by compounds that possess bulky lipophilic substituents in α position of the carboxylic acid and an aromatic group in the center which is substituted with one or two lipophilic residues.

6.2. Molecular pharmacological evaluation of PA derivatives and 2-(phenylthio)hexanoic acid derivatives as 5-LO inhibitors

Cellular LT formation is induced by Ca^{2+} mobilization and/or phosphorylation of 5-LO by MK-2/3 or ERK and requires translocation of 5-LO to the nuclear membrane [66]. In addition, 5-LO activity is modulated by a variety of factors that can significantly affect the potency of pharmacological inhibitors [262]. Regarding the complex regulation of 5-LO, it is very helpful to subject novel compounds to diversified experimental settings within *in vitro* studies in order to estimate their behavior *in vivo.* Moreover, it is important to consider the selectivity of a certain test compound for 5-LO, since many potential drug candidates turned out to inhibit the iron-containing and redox-sensitive 12- and 15-LOs as well. However, the test comounds selected for pharmacological evaluation **25**, **39** and **136** solely reduced the amount of 5-H(p)ETE and LTB_4 in PMNL stimulated with 2.5 µM ionophore A23187 in presence of 20 µM AA, and only a slight reduction of 15-H(p)ETE was observed at higher concentrations of **86**. In addition, this exclusive inhibition of 5-LO product formation makes a direct interference of the compounds with the stimulus ionophore very unlikely.

Direct 5-LO inhibitors are grouped into redox-active compounds, iron ligand inhibitors, nonredox-type inhibitors and a fourth class of 5-LO inhibitors that are supposed to act in an allosteric manner [200]. From the chemical structures of the compounds, no iron-chelating qualities are apparent, and radical scavenging properties were excluded in a DPPH assay. Reversibility of the 5-LO inhibitory effect could be shown in wash-out experiments (10-fold dilution) suggesting a non-covalent interaction of the compounds with the enzyme. Nonredox-type 5-LO inhibitors possess quite diverse structures and act by competing with fatty acid binding sites of the enzyme. Because of the lipophilic acid character of PA derivatives and 2-(phenylthio)hexanoic acid derivatives, a competitive mode of action would be expected. Surprisingly, isolated 5-LO was poorly inhibited by the compounds at low concentrations of AA (< 10 µM), whereas a considerably enhanced potency could be observed at increased substrate concentrations up to 30 µM. In previous studies, a second, regulatory fatty-acid-binding site of 5-LO was suggested [91], [114], [90]. It is possible that besides acting as 5-LO substrate, higher amounts of AA can induce LT formation at this regulatory binding site far from the catalytic center, and PA derivatives as well as 2-(phenylthio)hexanoic acid derivatives might antagonize this effect. Alternatively, micelle or lipid vesicle formation may occur at higher concentrations of

AA and incorporation of the amphiphilic compounds would thus improve their access to 5-LO [287]. However, in intact PMNL, inclusion of 20 µM AA only slightly increased the efficacy of **39** and **25**, and almost no difference in the inhibitory curves with 20 µM AA or without AA were observed for **86** and **136**. Possibly, additional components or mechanisms in intact cells compensate the need for a certain amount of AA. However, unphysiologically high substrate concentrations (40 or 60 µM) impaired the inhibitory effect of all derivatives (but not of the control inhibitor BWA4C) in intact cells as well as in cell-free assays. Assuming that the compounds target a second binding site for AA, such high amounts of the fatty acid might finally displace them from 5-LO.

Previous studies showed that the effect of some nonredox-type 5-LO inhibitors is impaired upon phosphorylation of 5-LO by MAPKs as well as by elevated peroxide levels [154], [153]. This might explain the reduced *in vivo* efficacy of compounds like ZM 230487 since inflammatory reactions are often associated by increased phosphorylation events and excessive peroxide formation. While treatment of PMNL with ionophore A23187 leads to a massive Ca^{2+}-influx into the cells, the bacterial peptide fMLP (a pathophysiologically relevant stimulus) activates 5-LO via release of intracellular Ca^{2+} and phosphorylation by MK-2 and/or ERKs [81]. Therefore, the inhibitory effect of the compounds on LTB_4 formation was addressed in PMNL stimulated with ionophore as well as after stimulation of the cells with 1 µM fMLP upon priming with 1 µg/ml LPS. While the concentration-response curves of **39** and **25** were comparable under both conditions, **86** and **136** were more effective after stimulation with ionophore, suggesting that at least some of the PA derivatives and 2-(phenylthio)hexanoic acid derivatives are more efficient on 5-LO activated by Ca^{2+} rather than under conditions where phosphorylations by MAPK partially or fully activate 5-LO.

LOOHs did not influence the potency of PA derivatives and 2-(phenylthio)hexanoic acid derivatives, and the decreased activity of the compounds that was observed in homogenates from PMNL was not restored under reducing conditions. Cell stress induced activation of 5-LO, in contrast, led to a declined efficacy of all tested compounds even if it was less pronounced for **136**. Nevertheless, the results of this assay have to be seen critically since the iron-ligand inhibitor BWA4C was surprisingly less effective as well under these conditions.

Taken together, PA derivatives and 2-(phenylthio)hexanoic acid derivatives share only a few properties with nonredox-type 5-LO inhibitors and do not possess typical structures of this class, suggesting a different way of action.

Another possible point of attack for 5-LO inhibitors is the regulatory C2-like domain [210]. This site of the enzyme binds several stimulating factors like Ca^{2+}, PC vesicles and thus membranes, OAG and CLP [66]. Ca^{2+} is known to increase the affinity of 5-LO towards AA and LOOHs and promotes binding of the enzyme to PC in the nuclear membrane [66]. Depletion of Ca^{2+} in cell-free assays did not alter the profile of any of the tested compounds. Therefore, interference of the derivatives with the Ca^{2+} binding site within the C2-like domain of 5-LO can be rather excluded.

It was demonstrated in this study that PA derivatives and 2-(phenylthio)hexanoic acid derivatives are not able to inhibit 5-LO products any longer if cellular components are present. The identity of the components that reduce the efficacy in PMNL homogenates or their 100,000×g supernatants and in crude *E. coli* supernatants is unknown. Possibly, the derivatives are captured by released lipids or membrane fractions so that their binding to 5-LO is prevented. Alternatively, PC vesicles that attach to the C2-like domain of 5-LO could disturb the interaction of the compounds with the enzyme. Indeed, the inhibitory effect of the derivatives (in particular of **39**) was attenuated after addition of PC to partially purified 5-LO, whereas PS had only a weak influence on the efficacy of the compounds. Moreover, a significant decrease in the potency of **39** and especially of **86** and **136** was observed in presence of OAG in cell-free assays. OAG directly activates 5-LO activity in particular in the absence of Ca^{2+} seemingly by increasing the affinity of LOOHs to 5-LO [98]. Curiously, OAG barely altered the inhibitory curves of **86** and **136** in intact PMNL, whereas **39**, **25** and also the negative control BWA4C showed an impaired efficacy, which is not readily understood. Attempts to demonstrate an influence of the derivatives on the interaction between CLP and 5-LO failed, presumably due to technical reasons. Since 5-LO translocation upon adequate stimulation is mediated by the C2-like domain, interference with this site may implicate a lower tendency of 5-LO to attach to the nuclear envelope. Interestingly, the contrary effect was observed for PA derivatives as well as for 2-(phenylthio)hexanoic acid derivatives. After stimulation of PMNL with ionophore A23187 or fMLP, the amount of 5-LO at the nucleus increased when cells were pretreated with the compounds. Notably, additional experiments with **39** showed that the compound itself targets 5-LO to the nuclear membrane without the need of a cell stimulus. At first glance, this result seems to conflict with the inhibitory

action of the compound. However, the amphiphilic substance might anchor 5-LO in the nuclear membrane via the C2-like domain thus inducing conformational changes of the enzyme and/or alter its orientation towards FLAP so that conversion of AA is prevented. Induction of nuclear membrane association of 5-LO coupled to reduced 5-LO activity was also observed for testosterone [144] and for thiopyrano[2,3,4-c,d]indoles, a class of dual 5-LO/FLAP inhibitors [288]. However, nuclear redistribution of 5-LO in PMNL occurred only upon treatment with relatively high amounts of PA derivatives or 2-(phenylthio)hexanoic acid derivatives (10 to 30 µM), whereas 5-LO product formation was already reduced by half at submicromolar concentrations of the compounds, suggesting that redistribution is probably not the major cause for 5-LO inhibition in PMNL [287].

Unfortunately, efforts to selectively precipitate 5-LO from PMNL lysates or a preparation of the isolated enzyme using immobilized PA derivatives or 2-(phenylthio)hexanoic acid derivatives failed, and a direct interference could also not be confirmed by SPR studies. Presumably, the lipophilic character of both 5-LO and the derivatives cause excessive unspecific binding in these experiments, which makes it technically difficult to detect selective interactions using these techniques.

To estimate the *in vivo* potency of novel inhibitors of 5-LO product formation, their potency in human whole blood assay was addressed since in this test system, the efficacy of a compound can be significantly influenced by diverse factors such as binding to plasma proteins or degradation by enzymes present in the blood. Unfortunately, none of the applied PA derivatives or 2-(phenylthio)hexanoic acid derivatives was successful in whole blood. It is very likely that the compounds strongly bind to plasma proteins, in particular to albumin, because of their lipophilic character [289]. Nevertheless, i*n vivo* efficacy of PA derivatives was demonstrated in two well established animal models of inflammation. Thus, a single dose of 10 mg/kg **39** rendered mice substantially less sensitive to PAF-mediated lethal shock, and already doses of 1.5 mg/kg **37** or **22** significantly reduced the inflammatory reaction after injection of λ-carrageenan into the pleural cavity of rats. Both models have been shown to be related to LTs [266], [268].

Taken together, the results of these pharmacological investigations suggest that PA derivatives and 2-(phenylthio)hexanoic acid derivatives constitute a novel class of direct 5-LO inhibitors with a different mode of action than redox-, iron ligand- or nonredox-type inhibitors. Interference

of the compounds with the binding site for PC, OAG and CLP and/or with a second binding site for AA within the C2-like domain of 5-LO seems very likely. Importantly, certain negative aspects of other types of 5-LO inhibitors that hampered successful development in the past (e.g. lacking selectivity or dependence on reducing conditions) are not or hardly evident for PA derivatives and 2-(phenylthio)hexanoic acid derivatives. This favorable *in vitro* profile and the potent anti-inflammatory effect of the compounds in rat pleurisy and PAF-induced shock mouse models strongly motivate for further *in vivo* investigations.

6.3. Further targets of PA derivatives and 2-(phenylthio)-hexanoic acid derivatives

PA derivatives as well as 2-(phenylthio)hexanoic acid derivatives do not only interfere with 5-LO, but also exhibit a prominent inhibitory effect on mPGES-1 without significantly affecting COX enzymes. mPGES-1 is involved in excessive PGE_2 formation from COX-2 derived PGH_2 and both enzymes are upregulated concomitantly at sites of inflammation [290]. Drugs that selectively inhibit PGE_2 biosynthesis are believed to lack the unwanted cardiovascular side effects of COX-2 inhibitors and dual inhibition of mPGES-1 and 5-LO might offer a promising strategy for intervention with inflammatory diseases [291], [292], [293]. Nevertheless, it has to be mentioned that mPGES-1 inhibition has also been considered critical since its blockade allows redirection of the substrate PGH_2 to other PG synthases. The consequences of this imbalance have to be eluted in further investigations [294].

In a joint publication with us, the group of Prof. Dr. G. Schneider (Swiss Federal Institute of Technology, Zürich, Switzerland) identified potential ligand binding sites for dual 5-LO and mPGES-1 inhibitors by comparison of cavities on the two protein surfaces via computational analysis [261]. For 5-LO, these investigations suggest a surface cavity which is neither located at the active site nor within the C2-like domain, and in mPGES-1 the potential binding pocket lies at the center of the protein. A common feature of both pockets is an Arg-His-Met triangle with similar edge lengths. Automated ligand docking studies for several phenylalkyloxy substituted PA derivatives yielded high score values for the predicted cavities in both 5-LO and mPGES-1. In contrast, binding of the compounds to the active site of 5-LO is rather unlikely according to the obtained docking results. A His residue in the binding pocket of 5-LO might interact with one of the two phenylalkyloxy residues (and presumably also with the phenyl residue of other PA derivatives and 2-(phenylthio)-hexanoic acid derivatives) and interference of the carboxylic

group with Lys and Gln side chains was suggested. For mPGES-1, both phenyl residues possibly are in contact with His side chains and hydrogen-bridges might be formed between the carboxylic group and an Arg residue. Mutations studies with the mentioned amino acids of 5-LO and mPGES-1 would offer a good opportunity to verify the interaction of PA derivatives with the proposed binding sites.

5-LO and mPGES-1 are not the only targets of PA derivatives and 2-(phenylthio)-hexanoic acid derivatives. Thus, the compounds were primarily synthesized in order to develop novel agonists of PPAR receptors, important targets for the treatment of dyslipidemia and type 2 diabetes [238], [239]. The group of Prof. Dr. M. Schubert-Zsilavecz (University of Frankfurt) showed that exchange of one moiety at the pyrimidine core of α-substituted PA led to an increased activity of both PPARα and PPARγ. In contrast, introduction of two phenylalkyloxy-residues yielded selective agonists on PPARγ [240], and 2-(phenylthio)hexanoic acid derivatives exclusively influence PPARα [241]. Various studies suggested PPAR receptors also as anti-inflammatory targets [221], [295], [296] so that a synergistic effect of dual 5-LO/mPGES-1 inhibition together with PPAR agonism in the treatment of chronic inflammatory diseases might be possible.

Notably, PA derivatives with two phenylalkyloxy residues were shown to also inhibit γ-secretase, an enzyme that catalyzes the generation of A*β* peptides in the brain playing crucial roles in the development of Alzheimer’s disease [240]. Although no data about the blood-brain-permeability of the compounds are available yet, their lipophilic structure suggests a good penetration in the brain and *in vivo* efficacy against Alzheimer’s disease might be assumed.

Studies in our group revealed that quinolinic PA derivatives with α-alkyl substitution inhibit typical neutrophil functions such as HLE secretion and the formation of ROS (other PA derivatives or 2-(phenylthio)hexanoic acid derivatives have not been tested in this respect) [245]. Since these derivatives (in their acidic form) were also able to reduce fMLP-mediated mobilization of intracellular Ca^{2+} and activation of p38 MAPK (which was not influenced by **25, 39**, **86**, **136**), they possibly interfere with upstream mechanisms like certain G proteins, PLC isoenzymes or components of the PI3K cascade. This might explain the above-mentioned increased inhibitory effect on 5-LO product formation in intact cells as well.

6.4. Searching for additional targets of PA- and 2-(phenylthio)hexanoic acid derivatives by protein fishing experiments

Protein fishing experiments in PMNL lysates with an immobilized α-hexyl substituted quinolinic PA derivative (**24**) revealed quinone oxidoreductase NQO2 as a binding partner of the compound. The physiological role of this flavoenzyme has not been completely elucidated yet and inconsistent results have been published regarding its role in detoxification processes [278]. Activity assays with recombinant NQO2 revealed a rather poor inhibitory effect of compound **24** on the enzyme so that physiological relevance of the binding is questionable.

Attempts to identify novel targets of α-alkyl substituted biphenylic PA derivatives or α-alkyl substituted 2-(phenylthio)hexanoic acid derivatives were not successful. In some experiments, identical bands were obtained for the α-alkyl substituted construct and its α-unsubstituted control although non ionic detergents in concentrations up to 1% were included in the fishing buffer. Presumably, the lipophilic compounds provoke a very high number of unspecific interactions. Moreover, separation of the fished proteins via one-dimensional gel electrophoresis might be insufficient. Electrophoretic separation via 2D-DIGE followed by fluorescence scanning yielded several spots exclusively for the α-alkyl substituted biphenylic **143**. However, the amount of bound proteins was too low to be detected with the rather insensitive silver staining method so that the spots could not be picked for MS detection.

7. Summary

LTs are proinflammatory lipids that mediate recruitment and activation of leukocytes, increase of vascular permeability and smooth muscle contraction. In addition, they seem to be involved in bone metabolism [297], the cardiovascular system, proliferation and (tumor) cell survival [298]. Therefore, pharmacological intervention with LT synthesis is a promising strategy to treat diseases like asthma, rheumatoid arthritis, psoriasis, allergic rhinitis, atherosclerosis and other cardiovascular diseases, osteoporosis and certain types of cancer. 5-LO catalyzes the first two steps in LT formation from liberated AA. The enzyme is composed of a catalytic domain at the C-terminus and an N-terminal C2-like domain that is able to bind a variety of regulatory factors, namely Ca^{2+}, PC vesicles, glycerides and CLP. Cellular activation of 5-LO by Ca^{2+} or certain MAPKs includes a translocation of the enzyme to the nuclear membrane where AA, liberated by $cPLA_2$ from phospholipids, is transferred to 5-LO with the help of FLAP. Moreover, the presence of LOOHs oxidizing the active site iron to the ferric form (Fe^{3+}) is required for 5-LO product formation.

Suppression of excessive 5-LO product formation can be achieved by different strategies. Besides inhibition of $cPLA_2$ or FLAP, direct interference with 5-LO has been subject of various studies during the last decades. Compounds that target the enzyme itself are categorized into redox-active compounds that uncouple the catalytic cycle by reducing the active site iron, iron ligand inhibitors, nonredox-type inhibitors that compete with AA or LOOH for binding to the enzyme, and structurally diverse 5-LO inhibitors, supposed to act in an allosteric manner. Despite strong efforts in the development of potential drug candidates, most of them failed due to a lack of efficacy in clinical studies or excited severe side effects such as methemoglobin formation, and zileuton is the only 5-LO inhibitor that has entered the market. However, since key roles of LTs in major diseases were demonstrated in numerous studies, the search for new concepts to interfere with 5-LO and LT biosynthesis is continuing.

This work presents a novel class of potent 5-LO inhibitors derived from the PPARα (and to a less extend PPARγ) agonist PA. The compounds were synthesized in the group of Prof. Dr. M. Schubert-Zsilavecz (University of Frankfurt) with the primary aim to develop highly active dual agonists of both PPARα and PPARγ. Structural variations that were beneficial for PPAR agonsim turned out to yield potent 5-LO inhibitors as well. Whereas PA itself did not influence LT

formation, introduction of a hexyl or a naphthyl residue in α-position of the carboxylic group as well as enlarged hydrophobic aromatic extension led to a substantial reduction of LT formation in human neutrophils but also in cell-free assays using partially purified 5-LO as enzyme source. For the most potent compounds **72** and **75**, IC_{50} values of 0.1 µM and 0.17 µM, respectively, were obtained in PMNL. In addition, a large series of compounds based on 2-(phenylthio)hexanoic acid, synthesized in the lab of Prof. Dr. M. Schubert-Zsilavecz, was subjected to SARs regarding 5-LO. For these derivatives, α-butyl substitution together with insertion of voluminous lipophilic residues were determinants for potent 5-LO inhibition with **136** as the most effective compound (IC_{50} = 0.9 µM in PMNL)-

Interestingly, the esters of PA and derivatives with α-alkyl chains up to n-butyl or with α-phenyl residues as well as esterified compounds with a quinoline or a 3-pyridylethylamine residue at the pyrimidine core showed a higher efficacy in intact neutrophils compared to their acidic analogs. In contrast, insertion of long and bulky alkyl/aryl groups in α-position and/or more hydrophobic moieties at the aromatic core of the molecule favored perpetuation of the free carboxylic acid. However, none of the esters was able to potently inhibit partially purified 5-LO.

The efficacy of many 5-LO inhibitors in intact cells as well as in cell-free assays strongly depends on the assay conditions. Therefore, this work investigated mechanistic aspects of four different types of PA- and 2-(phenylthio)hexanoic acid derivatives thereby providing a detailed analysis of their interference with the 5-LO enzyme.

Compounds with a free carboxylic group exert a direct and reversible inhibitory effect on 5-LO. Notably, slightly decreased IC_{50} values were obtained in PMNL compared to the isolated enzyme. This effect might be explained by additional points of attack of the compounds, leading to a suppression of 5-LO product formation in intact cells. A clear reduction of the potency of the derivatives was observed in presence of broken cell fractions (i.e. in PMNL homogenates or their 100,000×g supernatants and in crude *E. coli* supernatants). This suggests that either the compounds themselves interfere with cellular components or their interaction with 5-LO is somehow hampered in the presence of certain constituents released upon cell disruption. Therefore, the reduced efficacy on the partially purified enzyme might also result from remaining cellular components that could not be completely removed in the course of the purification process.

PA derivatives as well as 2-(phenylthio)hexanoic acid derivatives did not inhibit product formation from 12-LO, and only the bis-phenylethoxy substituted **86** slightly reduced 15-H(p)ETE formation so that a certain degree of selectivity of the compounds towards other LOs can be assumed. The chemical structures of the derivatives do not reveal any iron-chelating qualities, and radical scavenging activities were excluded. Moreover, their potency did not depend on reducing conditions as observed for classical nonredox-type 5-LO inhibitors.

Several results in this work indicate an interference of the derivatives with the regulatory C2-like domain of 5-LO. Thus, addition of OAG or PC (but not of PS), both factors that activate the enzyme via this site, attenuated the potency of the compounds. Moreover, a certain amount of AA was necessary to allow efficient suppression of partially purified 5-LO by PA- and 2-(phenylthio)hexanoic acid derivatives. Possibly, the compounds attach to a second binding site for AA (within the C2-like domain) that is involved in 5-LO regulation only at higher concentrations of substrate. Finally, upon addition of **39**, 5-LO translocated to the nuclear membrane in unstimulated neutrophils, which is presumably also mediated via the C2-like domain, although it is unclear if this contributes to the 5-LO inhibitory effect of the compounds.

PA derivatives and 2-(phenylthio)hexanoic acid derivatives did not only inhibit 5-LO product formation in PMNL treated with Ca^{2+}-ionophore A23187 but also after stimulation with LPS plus fMLP mimicking pathophysiological conditions in the body. Unfortunately, the compounds did not suppress LT biosynthesis in human whole blood, presumably due to their lipophilic acid character that causes a strong binding to plasma proteins. However, in two different animal models that are suitable for evaluation of LT synthesis inhibitors, the group of Prof. Dr. L. Sautebin (University of Naples, Italy) could show a potent anti-inflammatory effect of the compounds *in vivo*.

Recent data from literature suggest that simultaneous inhibition of both PGs and LTs is superior to interference with only one eicosanoid synthetic pathway. Thus, dual 5-LO/mPGES-1 inhibitors might possess a better safety profile in terms of gastrointestinal and cardiovascular risks than dual 5-LO/COX inhibitors. Interestingly, PA- as well as 2-(phenylthio)hexanoic acid derivatives are also able to efficiently block PGE_2 formation by mPGES-1 whereas COX enzymes are hardly affected. Moreover, it was shown in previous studies of our group that quinolinic PA derivatives with α-alkyl substitution (especially the free acids) suppress typical neutrophil functions such as

the formation of ROS and HLE secretion. This effect seems to be caused by an interference of the compounds with signaling cascades connecting fMLP stimulation with p38 MAPK and Ca^{2+} mobilization. In summary, the potent inhibitory effect on 5-LO and mPGES-1 together with the anti-neutrophilic properties strongly encourage for further pharmacological and pharmacokinetic studies in order to reveal a comprehensive profile of the compounds.

8. Zusammenfassung

Leukotriene (LTs) sind Arachidonsäure-Metabolite, welche eine wichtige Rolle bei Entzündungsreaktionen spielen. Sie bewirken eine Erhöhung der Gefäßpermeabilität und führen zur Kontraktion der glatten Muskulatur. Außerdem werden durch ihre Ausschüttung weitere Leukozyten angelockt und aktiviert. Ebenso konnte gezeigt werden, dass sie an der Regulation der Zellproliferation sowie am Knochenmetabolismus beteiligt sind. Des Weiteren üben sie gewisse Funktionen im kardiovaskulären System aus [297], [298].

Somit ist der pharmakologische Eingriff in die LT-Biosynthese ein vielversprechender Ansatz in der Behandlung zahlreicher entzündlicher und allergischer Erkrankungen wie z.B. Asthma, rheumatoider Arthritis, Psoriasis und allergischer Rhinitis, aber auch von Arteriosklerose und anderen kardiovaskulären Erkrankungen. Außerdem wird ein therapeutischer Effekt bei Osteoporose und verschiedenen Krebserkrankungen diskutiert.

Die ersten beiden Schritte der Umwandlung von freigesetzter Arachidonsäure (AA) zu LTs werden durch die 5-Lipoxygenase (5-LO) katalysiert, welche somit ein Schlüsselenzym der LT-Biosynthese darstellt. Die 5-LO besteht aus einer katalytischen Domäne am C-Terminus des Enzyms sowie einer regulatorischen n-terminalen C2-like Domäne. Letztere ist Bindungsstelle für einige Faktoren, welche die Enzymaktivität allosterisch beeinflussen wie Ca^{2+}, Phosphatidylcholin (PC), Glyceride und das coactosine-like protein (CLP). In intakten Zellen kann die 5-LO entweder durch Ca^{2+}-Bindung oder durch Phosphorylierung durch bestimmte Kinasen aktiviert werden, wobei das Enzym nach Zellstimulierung zur Kernmembran transloziert. Dort wird freigesetzte AA mithilfe des 5-LO-aktivierenden Poteins (FLAP) auf 5-LO übertragen. Außerdem ist für die Enzymaktivität eine Oxidation des nicht-hämgebundenen Eisens (Fe^{2+}) im katalytischen Zentrum durch Lipidhydroperoxide (LOOHs) nötig.

Eine Hemmung der 5-LO Produktbildung kann mithilfe verschiedener Strategien erreicht werden. Einerseits können die cyotsolische Phospholipase A_2 ($cPLA_2$) oder FLAP gehemmt werden, andererseits kann direkt Einfluss auf 5-LO genommen werden. In der Literatur wird zwischen vier Klassen von direkten 5-LO-Inhibitoren unterschieden: 1) redoxaktive Hemmstoffe, 2) Eisenligand-Inhibitoren, 3) Nichtredox-Inhibitoren und 4) eine Klasse an strukturell sehr unterschiedlichen Stoffen, welche die 5-LO wahrscheinlich allosterisch hemmen. Trotz

umfangreicher Bemühungen, einen potenten 5-LO-Hemmer zu entwickeln, ist bisher nur der Eisenligand-Inhibitor Zileuton als Arzneimittel zugelassen. Viele andere Substanzen konnten aufgrund von schweren Nebenwirkungen oder ungenügender Wirksamkeit nicht zur Marktreife gebracht werden. Dennoch stellt die 5-LO aufgrund ihrer Beteiligung an der Entstehung der sogenannten „Volkskrankheiten" ein sehr interessantes Target dar und die Suche nach effektiven und sicheren Hemmstoffen ist eine große Herausforderung.

In dieser Arbeit wird eine Klasse neuartiger 5-LO-Inhibitoren vorgestellt, welche sich strukturell von Pirinixinsäure (PA), einem Peroxisom-Proliferator-aktivierten Rezeptor (PPAR)α-Agonisten, ableiten. Die Substanzen wurden in der Arbeitsgruppe von Prof. Dr. M. Schubert-Zsilavecz an der Universität Frankfurt synthetisiert, in erster Linie um potente duale PPARα/γ-Agonisten zu entwickeln. Es stellte sich heraus, dass durch strukturelle Veränderungen, welche einen positiven Einfluss auf den PPAR-Agonismus zeigten, auch eine Hemmung der 5-LO-Produktbildung in Ca^{2+}-Ionophor-stimulierten humanen polymorphnukleären Leukozyten (PMNL) sowie an rekombinanter 5-LO erreicht werden konnte. Während die LT-Biosynthese durch PA selbst nicht beeinflusst wurde, resultierte aus der Einführung von Hexyl- oder Naphtylresten in α-Position der Carboxylgruppe, wie auch aus der Vergrößerung des hydrophoben aromatischen Molekülteils, eine signifikante 5-LO-Hemmwirkung. Für die potentesten Substanzen **72** und **75** wurden IC_{50}-Werte von 0.1 µM bzw. 0.17 µM in PMNL ermittelt. Darüber hinaus wurde im Labor von Prof. Dr. M. Schubert-Zsilavecz eine Reihe von Substanzen synthetisiert, welche sich von 2-(Phenylthio)hexansäure ableiten und ebenfalls potente Inhibitoren der 5-LO darstellen. In dieser Substanzgruppe lieferten α-butyl-substituierte Moleküle mit voluminösen lipophilen Resten die besten Ergebnisse hinsichtlich Hemmung der LT-Biosynthese, wobei Substanz **136** die effektivste Wirkung zeigte (IC_{50} = 0.9 µM in PMNL).

Interessanterweise zeigten Ester von PA-Derivaten mit α-Alkylsubstituenten von bis zu vier C-Atomen, Ester von PA-Derivaten mit α-Phenylsubstituenten sowie Ester von Substanzen, welche über einen Chinolinrest oder einen 3-Pyridylethylaminrest am Pyrimidinkern verfügen, eine deutlich höhere Wirksamkeit in zellulären Assays als ihre nicht-veresterten Analoga. Im Gegensatz dazu war die freie Säuregruppe essentiell für Substanzen mit langkettigen oder sperrigen Alkyl-/Arylgruppen in α-Position und/oder hydrophoben Resten im anderen Molekülteil. Allerdings konnte durch veresterte Substanzen keine potente Hemmwirkung an isolierter 5-LO erreicht werden. Die Säurefunktion, welche diesen lipophilen Molekülen große

Ähnlichkeit mit AA verleiht, scheint für eine direkte Interaktion mit dem Enzym somit essentiell zu sein, wohingegen die potente LT-Biosynthesehemmung der Ester mit kürzeren und eher hydrophilen Substituenten möglicherweise durch Interaktion mit anderen zellulären Mechanismen vermittelt wird.

Die Wirksamkeit vieler 5-LO-Inhibitoren in intakten Zellen wie auch in zellfreien Systemen ist stark von den gewählten Versuchsbedingungen abhängig. In dieser Arbeit wurden vier ausgewählte Vertreter der PA- bzw. 2-(Phenylthio)hexansäurederivate unter verschiedensten Assaybedingungen getestet, um ein detailliertes Bild der Interaktion dieser Substanzen mit der 5-LO zu erhalten. Derivate, welche über eine freie Säuregruppe verfügen, üben eine direkte und reversible Hemmwirkung auf 5-LO aus, wobei die IC_{50}-Werte in PMNL im Vergleich zum isolierten Enzym etwas niedriger liegen. Dieser Effekt könnte auf zusätzliche Angriffspunkte der Substanzen hindeuten, deren Beeinflussung ebenfalls eine Erniedrigung der 5-LO-Produktbildung zur Folge hat. Eine signifikant schlechtere Wirksamkeit der Derivate wurde in Anwesenheit von Zellbestandteilen, wie sie in PMNL-Homogenaten oder ihren 100.000×g-Überständen sowie im Überstand von *E. coli*-Lysaten vorkommen, beobachtet. Dies könnte entweder durch eine direkte Wechselwirkung der Derivate mit zellulären Bestandteilen oder durch eine Störung der Interaktion zwischen Substanz und Enzym durch bestimmte Stoffe, welche bei der Zerstörung von Zellen anfallen, bedingt sein. Somit könnte die verringerte Potenz der Derivate an isolierter 5-LO auch durch verbliebene Zellbestandteile, welche durch den Aufreinigungsprozess nicht vollständig entfernt werden konnten, verursacht sein.

Sowohl PA- als auch 2-(Phenylthio)hexansäurederivate waren nicht in der Lage, die 12-LO-Produktbildung zu hemmen und nur das bis-phenylethoxy-substituierte Derivat **86** hatte einen schwach inhibierenden Einfluss auf die 15-Hydroperoxyeicosatetraen-säure (15-H(p)ETE) Bildung, so dass man von einer selektiven Hemmung der 5-LO ausgehen kann. Die chemischen Strukturen der Substanzen lassen keine Funktion als Eisenchelator erkennen und in dieser Studie wurde gezeigt, dass die Derivate keine Radikalfängereigenschaften besitzen. Im Gegensatz zu den klassischen Nichtredox-Inhibitoren hemmen PA- und 2-(Phenylthio)hexansäurederivate die 5-LO auch unter nicht-reduzierenden Bedingungen.

Einige Ergebnisse dieser Arbeit deuten auf eine Interaktion der Substanzen mit der regulatorischen C2-like Domäne der 5-LO hin. So wurde die Wirksamkeit der Derivate durch 1-

Oleoyl-2-acetyl-sn-glycerol (OAG) oder PC (jedoch nicht Phosphatidylserin (PS)), Faktoren, welche an dieser Stelle des Enzyms binden, abgeschwächt. Des Weiteren wurde partiell aufgereinigte 5-LO erst in Anwesenheit einer bestimmten Menge an AA effektiv gehemmt. Möglicherweise binden die Substanzen an eine zweite, regulatorische Bindungstelle für AA innerhalb der N-terminalen Domäne, die erst ab einer gewissen Substratkonzentration relevant wird. In PMNL kam es nach Behandlung mit **39** zu einer Translokation der 5-LO zur Kernmembran, ohne dass ein weiterer Stimulus benötigt wurde. Dieser Effekt wird wahrscheinlich ebenfalls über die C2-like Domäne vermittelt.

PA- und 2-(Phenylthio)hexansäurederivate waren nicht nur in der Lage, die 5-LO-Produktbildung in Ca^{2+}-Ionophor-stimulierten PMNL zu hemmen, sondern auch in Zellen, welche mit Lipopolysaccharid (LPS) und N-Formylmethionyl-Lencyl-Phenylalanin (fMLP), einem pathophysiologisch relevanten Stimulus, behandelt wurden. Leider konnte durch die Substanzen keine Hemmung der LT-Biosynthese in menschlichem Vollblut gezeigt werden. Der Grund hierfür liegt wahrscheinlich am lipophilen Säurecharakter der Derivate, wodurch es zu einer starken Bindung an Plasmaproteine kommen kann. Dennoch konnte in der Arbeitsgruppe von Prof. Dr. L. Sautebin (Universität Neapel, Italien) anhand von zwei Tierversuchsmodellen, welche mit einer vermehrten LT-Bildung assoziiert sind, gezeigt werden, dass die Derivate einen sehr potenten anti-inflammatorischen Effekt *in vivo* ausüben und dabei die LTB_4-Bildung hemmen.

Neuere Literaturdaten deuten auf einen synergistischen Effekt bei gleichzeitiger Hemmung der Prostaglandin (PG)- und LT-Synthese hin und duale Inhibitoren von 5-LO und mikrosomale Prostaglandin E2 Synthase (mPGES)-1 scheinen den dualen Cyclooxygenase (COX)/5-LO-Hemmern in Bezug auf das gastrointestinale und kardiovaskuläre Risiko überlegen zu sein. Interessanterweise sind sowohl PA- als auch 2-(Phenylthio)hexansäurederivate in der Lage, die PGE_2-Bildung durch die mPGES-1 effizient zu hemmen, wohingegen COX-Enzyme kaum beeinflusst werden. Außerdem wurde in früheren Untersuchungen unserer Arbeitsgruppe gezeigt, dass Chinolin-tragende PA-Derivate mit α-Alkylsubstitution (insbesondere die freien Säuren) in der Lage sind, typische Funktionen von Neutrophilen wie zum Beispiel die Bildung reaktiver Sauerstoffspezies oder die Sekretion der humanen Leukozytenelastase zu hemmen. Dieser Effekt folgt wohl aus der Interaktion der Substanzen mit Signalkaskaden, welche nach Stimulierung durch fMLP zu einer Aktivierung von p38Mitogen Aktivierter Protein-Kinase (MAPK) und zur

Mobilisierung von Ca^{2+} führen. Schließlich ermutigen die potente Hemmwirkung auf die 5-LO und die mPGES-1 zusammen mit den anti-neutrophilen Eigenschaften weitere pharmakologische und pharmakokinetische Studien durchzuführen, um ein umfangreiches Bild der Substanzen zu erhalten.

9. References

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10. Publications

Original papers

Hieke, M.[*], Greiner, C.[*],, Dittrich, M., Reisen, F., Schneider, G., Schubert-Zsilavecz, M., Werz, O. (2011) *Discovery and biological evaluation of a novel class of dual microsomal prostaglandin E2 synthase-1/5-lipoxygenase inhibitors based on 2-[(4,6-diphenethoxypyrimidin-2-yl)thio]hexanoic acid.* J Med Chem. Accepted Manuscript, DOI: 10.1021/jm200092b
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Greiner, C., Zettl, H., Koeberle, A., Pergola, C., Northoff, H., Schubert-Zsilavecz, M., Werz, O. (2011) *Identification of 2-mercaptohexanoic acids as dual inhibitors of 5-lipoxygenase and microsomal prostaglandin E2 synthase-1.* Bioorg Med Chem. 19(11); 3394-401.

Greiner, C., Hornig, C., Rossi, A., Pergola, C., Zettl, H., Schubert-Zsilavecz, M., Steinhilber, D., Sautebin, L., Werz, O. (2011) *2-(4-(Biphenyl-4-ylamino)-6-chloropyrimidin-2-ylthio)octanoic acid (HZ52) - a novel type 5-lipoxygenase inhibitor with favorable molecular pharmacology and efficacy in vivo.* Br J Pharmacol.“Accepted Article”; doi: 10.1111/j.1476-5381.2011.001451.x

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Poster presentations

Greiner, C., Dehm, F., Pergola, C., Dittrich, M., Zettl, H., Rossi, A., Hörnig, C., Steinhilber, D., Schubert-Zsilavecz, M., Sautebin, L., Werz, O. (2010) *Inhibition of 5-Lipoxygenase by Derivatives of Pirinixic Acid: Molecular Pharmacology and Efficacy in Vivo.* Poster presentation at 3rd European Workshop on Lipid Mediators, Pasteur Institute - Paris, June 3-4, 2010

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