Identification, characterization and evaluation of novel leukotriene biosynthesis inhibitors

Identifizierung, Charakterisierung und Bewertung neuartiger Hemmstoffe der Leukotrienbiosynthese

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Contents

Abbreviations

12-HHT	12(S)-hydroxy-5-cis-8,10-trans-heptadecatrienoic acid
5-HEDH	5-hydroxyeicosanoid dehydrogenase
5-oxo-ETE	5-oxo-eicosatetraenoic acid
5α-DHT	5α-dihydrotestosterone
A23187	Ca^{2+}- ionophore
AA	arachidonic acid
ADP	adenosine diphosphate
ALA	α-linolenic acid
AMP	adenosine monophosphate
APS	ammonium persulfate
ASA	acetylsalicylic acid (Aspirin®)
ATL	aspirin-triggered lipoxin
ATP	adenosine triphosphate
BAPTA-AM	1,2-bis-(o-Aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid,tetraacetoxymethyl ester
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
C5a	complement component 5a
cAMP	cyclic adenosine monophosphate
CLP	coactosin-like protein
CTP	cytosine triphosphate
COPD	chronic obstructive pulmonary disease

COX	cyclooxygenase (syn. PGH_2 synthase)
$cPLA_2$	cytosolic phospholipase A_2
CYP	cytochrome P450
cys-LT	cysteinyl-leukotrienes
DAG	diacylglycerol
DHA	docosahexaenoic acid
DMSO	dimethyl sulfoxide
DPPH	di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (IUPAC)
DTT	dithiothreitol
EET	epoxyeicosatrienoic acid
EIA	enzyme immunoassay
EPA	eicosapentaenoic acid
ERK	extracellular-regulated kinase
FLAP	five-lipoxygenase activating protein
fMLP	N-formylmethionyl-leucyl-phenylalanine
GPCR	G-protein coupled receptor
GPx	glutathione peroxidase
GSH	glutathione
GTP	guanosine triphosphate
H(P)ETE	hydro(peroxy)-6,8-trans-11,14-cis-eicosatetraenoic acid
HPLC	high perfomance liquid chromatography
i.p.	intraperitoneal
IL-1β	interleukin-1β

IPTG	isopropyl-β-D-1-thiogalactopyranoside
kB	kilobase
LA	linoleic acid
LB	lysogeny broth (Luria Bertani)
LO	lipoxygenase
LOOH	lipid hydroperoxide
LPS	lipopolysaccharide
LT	leukotriene
LTA_4	leukotriene A_4 5(S)-trans-5,6,-oxido-7,9-trans-11,14-cis-eicosatetraenoic acid
LTA4H	leukotriene A_4 hydrolase
LTB_4	leukotriene B_4 5(S),12(R)-dihydroxy-6,8,10,14-eicosatetraenoic acid
LTC_4	leukotriene C_4
LTC4S	leukotriene C_4 synthase
LUV	large unilamellar vesicle
LX	lipoxin
MAPEG	membrane-associated protein in eicosanoid and glutathione metabolism
MAPK	mitogen-activated protein kinase
MGST	microsomal glutathione S-transferase
MK	mitogen-activated protein kinase-activated protein kinase
MKP	mitogen-activated protein kinase (MAPK) phosphatase
MLV	multilamelar vesicle
mPGES-1	microsomal prostaglandin E_2 synthase-1

NADPH	nicotinamide adenine dinucleotide phosphate
NBT	nitro blue tetrazolium chloride
NDGA	nordihydroguaiaretic acid
NES	nuclear export sequence
NLS	nuclear localization sequence
NP-40	Nonidet-P40 ((*p-tert*-Octylphenoxy) polyethoxyethanol)
NSAID	nonsteroidal anti-inflammatory drug
OAG	1-oleoyl-2-acetyl-sn-glycerol
PAF	platelet-activating factor
PA-P	phosphatidic acid phosphatase
PAPC	1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphocholine
PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PGB_1	prostaglandin B_1
PGE_2	prostaglandin E_2
PGHS	prostaglandin H synthase
PGI	prostacyclin
PKA	proteinkinase A
PKC	proteinkinase C
PLD	phospholipase D
PMNL	polymorphonuclear leukocytes
PMSF	phenylmethyl sulfonyl fluoride

POG	1-palmitoyl-2-oleoyl-sn-glycerol
PPAR	peroxisome proliferator-activated receptor
PRP	platelet-rich plasma
PUFA	polyunsaturated fatty acid
ROS	reactive oxygen species
RP	reversed phase
RT	room temperature
RXR	retinoid X receptor
SDS	sodiumdodecylsulfate
SDS-PAGE	sodiumdodecylsulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SRS-A	slow reacting substance of anaphylaxis
STI	soybean trypsine inhibitor
TBS	tris buffered saline
TEA	triethanolamine
TFA	trifluoroacetic acid
TGF-β	transforming growth factor-β
TNF-α	tumor necrose factor-α
Tris	Tris(hydroxymethyl)-aminomethan
TX	thromboxane
VDR	vitamin D receptor
VDRE	vitamin D response element
w/o	without

Introduction

1.1 Polyunsaturated fatty acids in health and disease

Among the fatty acids, long-chain polyunsaturated fatty acids (PUFA) play an important role in many physiological processes. Since they cannot be synthesized *de novo*, they have to be consumpted from dietary sources. They are classified according to the location of the first double bond, seen from the methyl end (ω-end) of the carbon chain, resulting in ω -3 and ω -6 fatty acids **(Fig. 1).**

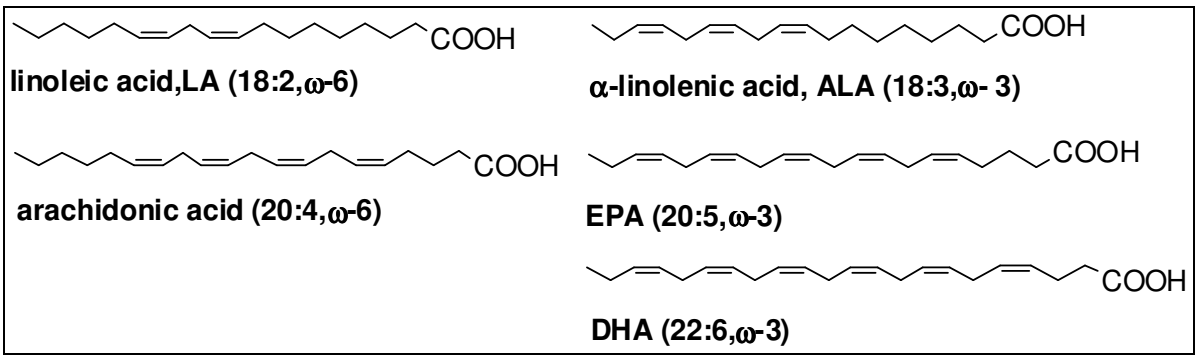


Fig. 1 Omega-3 and Omega-6- PUFAs

α-Linolenic acid (ALA) is a plant-derived, essential ω-3 fatty acid and can be converted to eicosapentaenic acid (EPA) and docosahexaenoic acid (DHA) in the body. However, the conversion rates are quite low [1]. Epidemiological studies in a population of Greenland eskimos and japanese people in the early 1980s revealed the nutritional importance of ω-3 fatty acids. Over the years, various studies confirmed that consumption of seafood containing high amounts of EPA and DHA, reduced the incidence of cardiovascular diseases and inflammatory disorders [2]. After oral application of EPA and DHA (1-3 g/day), triacylglycerol and cholesterol levels were decreased [3] and fish oil intake at doses up to 5 g/day showed anti-inflammatory effects in the treatment of rheumatoid arthritis [4]. EPA and DHA are precursors for the biosynthesis of important anti-inflammatory lipid mediators termed resolvins and protectins [5].

Moreover, a protective aspect for ω-3 PUFAs in Alzheimer's disease [6], [7] and a benefit in the treatment of psychiatric disorders [8] is discussed.

Linoleic acid (LA), mainly occurring in vegetable oils (e.g. corn, sunflower) is an important ω-6-PUFA, contributing around 90% to the dietary intake of ω-6-PUFAs. In mammalian cells, the ω-6-PUFA arachidonic acid (AA) is generated from LA by enzymatic elongation and desaturation [9]. After incorporation in membrane phospholipids, AA serves as starting substance for the synthesis of eicosanoids. The name originates from the number of carbon atoms (Greek: eicosa = twenty for 20-carbon fatty acid). The (patho)physiological roles of eicosanoids derived from AA, in particular prostaglandins (PGs) and leukotrienes (LTs), drawed the attention of researchers for more than 40 years now [10].

1.2 Eicosanoid biosynthesis

The term eicosanoids describes a class of biologically active lipid mediators derived from twenty-carbon PUFAs. AA (C20:4,ω-6) represents the major precursor for the synthesis of eicosanoids. It starts with the cleavage of AA from membrane phospholipids by cytosolic phospholipase A_2 ($cPLA_2$) followed by conversion via several enzymatic and non-enzymatic routes (**Fig. 2**).

The lipoxygenase (LO) pathway converts AA to leukotrienes (LTs), hepoxilins and lipoxins (LXs). Lipoxygenases (LOs) are iron-containing dioxygenases that catalyze the stereo-specific insertion of molecular oxygen into PUFAs. The animal LOs are classified according to the positional specifity after oxygenation of AA [11] (for details, see **1.5).**

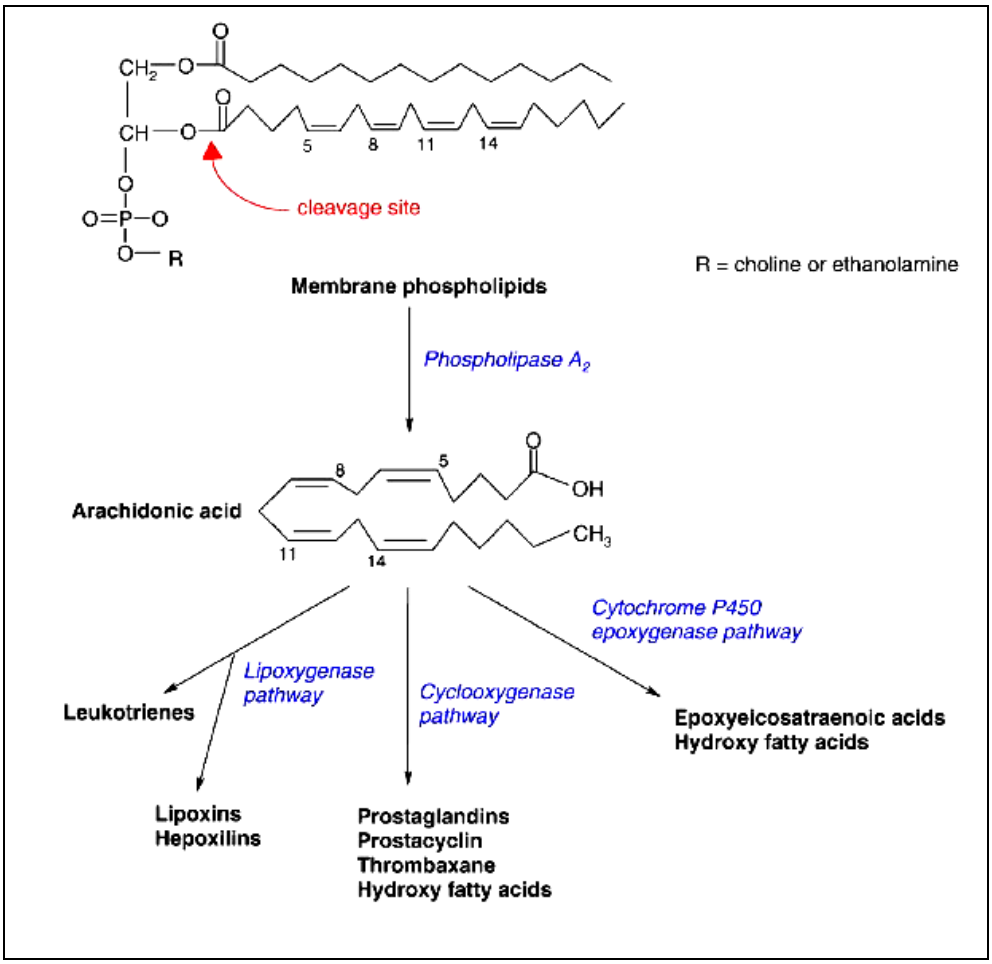


Fig. 2: Eicosanoid biosynthesis [12]

Conversion of AA by cyclooxygenases (COX) and further conversion by specific prostanoid synthases results in PGs, prostacyclins (PGIs) and thromboxanes (TXAs), summarized as prostanoids. In humans, COX-1 as a constitutively expressed enzyme and COX-2, induced by inflammatory stimuli are responsible for the generation of the endoperoxide PGH_2 which is then further converted by specific synthases to prostanoids (for details, see chapter **1.4**).

Alternatively, cytochrome P450 (CYP)-epoxygenases and ω-hydroxylases convert AA to epoxyeicosatrienoic acids (EETs) and hydroxyeicosatetraenoic acids (HETEs). The CYP metabolites possess high relevance for renal and cardiovascular functions [13]. Moreover, EETs activate Ca^{2+}-activated K^+-channels resulting in vascular relaxation and were ascribed to have anti-

inflammatory effects [14]. In contrast, the expression of CYP ω-hydroxylase in tumor cells and a pro-angionetic action of EETs implicates a role in carcinogenesis [12], [15].

The biological actions of the eicosanoids are mediated via G-protein-coupled receptors (GPCR). Their wide-ranging actions are regulated by different coupling to G-proteins, the cellular distribution of the enzymes and expression of the receptors on the cells. Eicosanoids play pathophysiologial roles in acute inflammation, cardiovascular diseases, cancer [16], atherosclerosis and many other inflammatory diseases [17]. In contrast, anti-inflammatory effects for AA metabolites were described as seen for lipoxins and related metabolites.

Thus, the eicosanoid pathway offers a multitude of interventions such as the inhibition of synthesis of pathophysiologic mediators or by intervention with their biological actions at their receptors.

1.3 Phospholipase A_2 (PLA_2)

The level of free AA in mammalian cells is a strictly controlled process. AA itself functions as an activator of ion channels and NADPH oxidase, resulting in the “oxidative burst” and in induction of apoptosis [18]. In inflammatory cells, AA obtained either by biosynthesis from essential fatty acids or by dietary intake is esterified to the sn-2 position of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) [19] and low levels of unesterified AA are present. Thus, the amount of free AA is determined by the balance between the incorporation of AA to phospholipids by CoA-dependent acyl transferases and transacylases and concomitant deacylation by phospholipases (“Lands cycle”). Upon cellular stimulation, the cycle is shifted towards the deacylation of AA, resulting in enzymatic cleavage of AA from the phospholipid.

PLA_2s catalyze the hydrolysis of the sn-2 ester bond of glycerophospholipids, resulting in a free fatty acid and a lyso-phospholipid [20]. More than 15 groups of phospholipase A_2 are known, subdivided in five types: secreted sPLAs

($sPLA_2$), cytosolic cPLA2s ($cPLA_2$), Ca^{2+}-independent PLA_2s ($iPLA_2$), platelet activating factor acetyl hydrolases (PAF-AH) and lysosomal PLA_2s [21].

Group IVA PLA_2 ($cPLA_2\alpha$) is specific for the cleavage of esterified AA in sn-2 position. The active site contains a catalytic dyad composed of Ser228 and Asp549. $cPLA_2\alpha$ binds two Ca^{2+}-ions at a N-terminal C2 domain [22]. An increase of the intracellular Ca^{2+} concentration leads to binding of Ca^{2+} at the C2 domain followed by translocation of the enzyme to the nuclear membrane and the endoplasmic reticulum. In addition, phosphorylation of $cPLA_2\alpha$ at Ser505 by MAPK regulates the activity of $cPLA_2\alpha$ [23]. Further regulatory mechanisms involve interactions with the lipid mediators ceramide-1-phosphate (C1P), or phosphatidylinositol (4,5) bisphosphate (PIP_2) [20]. The important role of $cPLA_2\alpha$ in eicosanoid production and inflammatory processes was investigated in $cPLA_2\alpha$-deficient knockout mice, where deletion of the $cPLA_2\alpha$ gene led to significant decrease in PG and LT formation and a reduced allergic response [24].

1.4 Prostanoids

Prostanoids are a family of lipid mediators arising from the oxygenation of AA by prostaglandin H synthases (PGHS), commonly referred to as COX. In mammals, COX exists in two isoforms: COX-1 is widely expressed in tissues and cells and is considered as “housekeeping” enzyme and COX-2, which is induced in response to certain inflammatory stimuli and in tumor development. However, a constitutive expression of COX-2 is also well-known in brain, kidney and the female reproductive tract [25]. The existence of COX-3 was assumed, but it was later identified as a splice variant of COX-1 [26], [27].

COXs are important pharmacological targets of the widely used nonsteroidal anti-inflammatory drugs (NSAIDs) in the treatment of fever, pain, rheumatic diseases and inflammatory conditions [28]. COX-1 and -2 convert AA to the endoperoxide PGG_2 by introduction of oxygen at C-15 (cyclooxygenase reaction) followed by reduction of the hydroperoxide to the corresponding

alcohol PGH_2 (peroxidase reaction) [29]. PGH_2 serves as substrate for cell-specific downstream synthases and isomerases, yielding PGs, PGIs or TXs (**Fig. 3**).

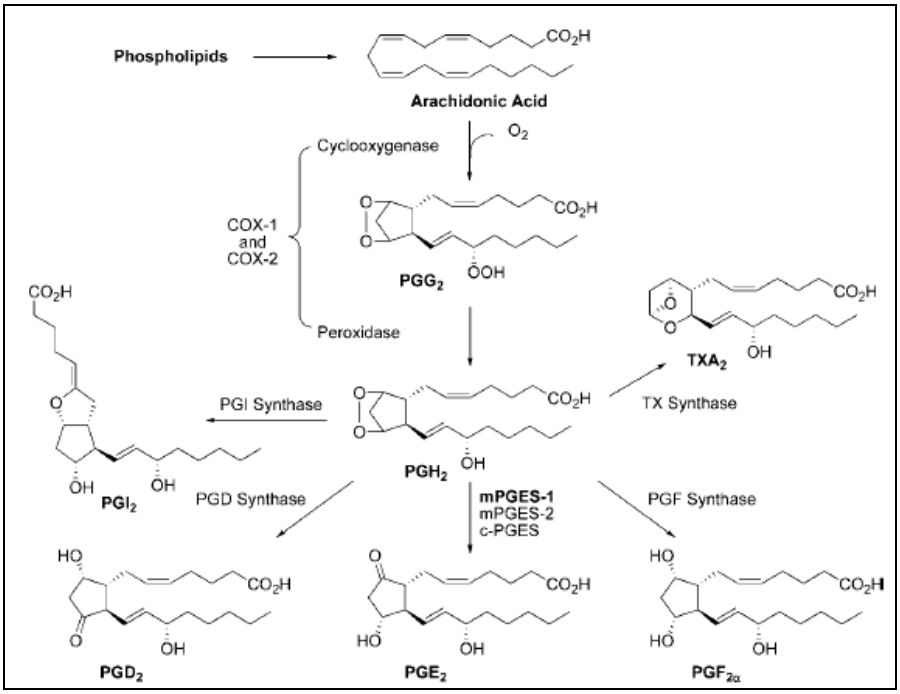


Fig. 3 Prostanoid biosynthesis [30]

The biological effects of prostanoids are mediated at specific GPCR and their functional roles were analyzed by targeted deletion of the receptors [31]. 10 subtypes have been described so far: four receptor subtypes for PGE_2 (EP_1-EP_4), two PGD_2 receptors (DP_1/DP_2), two for TXA_2 (TP_α/TP_β) and for $PGF_{2\alpha}$ (FP_A and FP_B) and PGI_2 (IP) [32]. Additionally, PGE_2 interacts with the PPARδ receptor. TXA_2 has strong vasoconstrictory and thrombogenic properties and exerts its functions mainly in platelets. PGI_2 acts as physiological antagonist of TXA_2 and possesses vasodilatatory and inhibits platelet aggregation [33].

PGI_2 and PGE_2 contribute to the protection of the gastric mucosa by increase of mucosal blood flow and stimulate bicarbonate secretion [34]. Depending on the EP receptor subtype, PGE_2 was linked to osteoclastogenesis, development of fever and pain as well as to the regulation of blood pressure in the kidney. $PGF_{2\alpha}$ plays an important role in the reproductive tract at parturition and onset

of labor; PGD_2 is generated by mast cells in allergic diseases [31]. Additionally, PGI_2, TXA_2 and $PGF_{2\alpha}$ influence renal functions [32].

The use of specific COX-2 inhibitors ("Coxibs") was shown to reduce the unwanted gastrointestinal side effects of non-selective NSAIDs such as ibuprofen or diclofenac. However, a long-term-use of COX-2 inhibitors was related to higher risk of cardiovascular side effects such as myocardial infarction or cerebrovascular events [35]. The cardiovascular side effects of the Coxibs were ascribed to reduction of COX-2-derived antithrombotic PGI_2, leading to an imbalance and an excess of pro-aggregating TXA_2 resulting in cardiovascular events [36].

COX-2 is functionally coupled to the downstream microsomal PGE_2 synthase-1 (mPGES-1). mPGES-1 catalyzes the conversion of COX-2 derived PGH_2 to PGE_2, which is considered as the major PG involved in inflammation, pain, arthritis and cardiovascular diseases [30]. Upon exposure to proinflammatory cytokines such as LPS, TNF-α or IL-1β, mPGES-1 expression is strongly upregulated [37]. Suppression of mPGES-1-derived PGE_2 formation is supposed to have beneficial effects in various inflammatory diseases, cancer [38] and cardiovascular diseases [39]. A genetic knockout of mPGES-1 in a mouse model confirmed the role of mPGES-1 in cancer treatment [40]. Thus, selective inhibition of mPGES-1 represents a novel and specific therapeutic approach for anti-inflammatory therapy [30].

1.5 Mammalian lipoxygenases

Mammalian LOs are classified based on the phylogenetic aspect into epidermis-type-LO, 5-LO, platelet-type 12-LO and 12/15-LO. In humans, five homologues were identified: the 5-(S)-LO, platelet-type 12-(S)-LO, epidermis-type 12(R)-LO, reticulocyte type 15-(S)-LO (15-LO-1) and epidermis-type 15(R)-LO (15-LO-2) [41].

However, the nomenclature of the enzymes according to the positional specificity of the oxygenated LO products is not fixed. For example, the

mammalian reticulocyte-type 15-LO forms a mixture of C-12- and C-15 oxygenated products [42]. Therefore, this enzyme is designated as 12/15-LO. Mammalian LOs consist of a single polypeptide chain with 662-676 amino acids with a molecular mass of 75-80 kDa. The catalytic domain of LOs contains a single non-heme iron atom surrounded by four conserved histidine residues and an isoleucine.

The oxygenase reaction of LOs starts with the stereoselective abstraction of hydrogen from the fatty acid, resulting in a pentadienyl radical, followed by rearrangement of the radical electron either in the direction of the methyl or the carboxylate end (**Fig. 4**). Then, oxygen is inserted at the antarafacial site of the hydrogen abstraction, resulting in a peroxy radical fatty acid. This peroxy radical is reduced by an electron from the catalytic iron which is reoxidized to its ferric (Fe^{3+}) form.

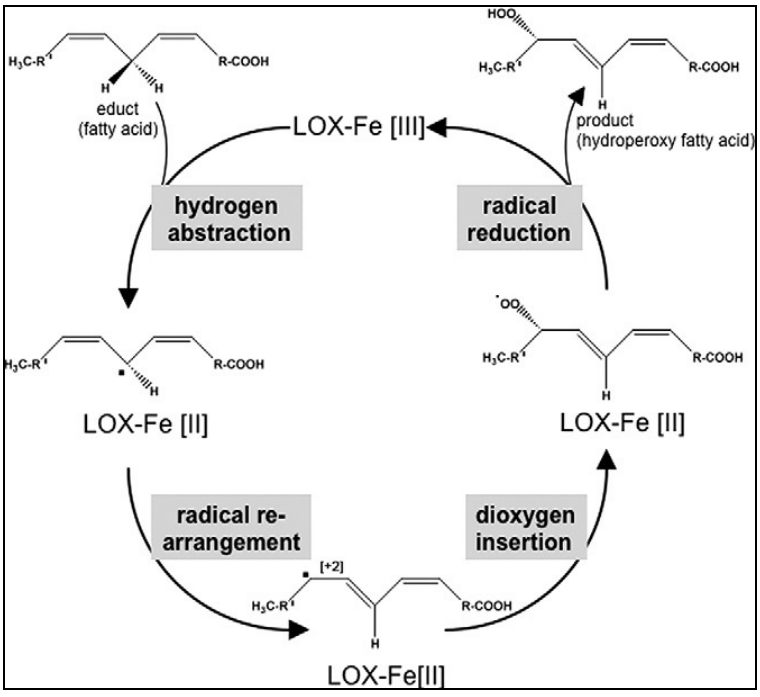


Fig. 4 Mechanism of the catalytic lipoxygenase reaction [11]

The positional specificity of mammalian 5-, 12- and 15-LOs depends on the volume of the active site. The alkyl end of AA binds in the bottom of the cleft and the acid end interacts with a basic amino acid such as arginine (12- and 15-

LO) or lysine (5-LO) [43]. Moreover, the orientation of the fatty acid seems to determine the position where the oxygenation occurs [44].

Genetic disruption of LO genes provided insights to the different roles of the mammalian LOs. 5-LO-deficient mice showed reduced signs of inflammation in typical models. In addition, 5-LO plays a role in host defense response against to airway infection with Klebsiella pneumoniae. A genetic knockout of platelet-type-12-LO was linked to thrombotic diseases, tumor development and maintenance of the epidermal water barrier. Studies with 15-LO-deficient mice mouse showed a clear role in atherosclerosis and possible involvement in type 1 diabetes and cardiovascular diseases [45].

1.6 Lipoxygenase interaction products (lipoxins)

The combined action of 5-, 12- and 15-LOs by cell-cell interactions and transcellular biosynthesis of lipid mediators leads to the formation of an important class of anti-inflammatory mediators, the lipoxins (LXs) [46].

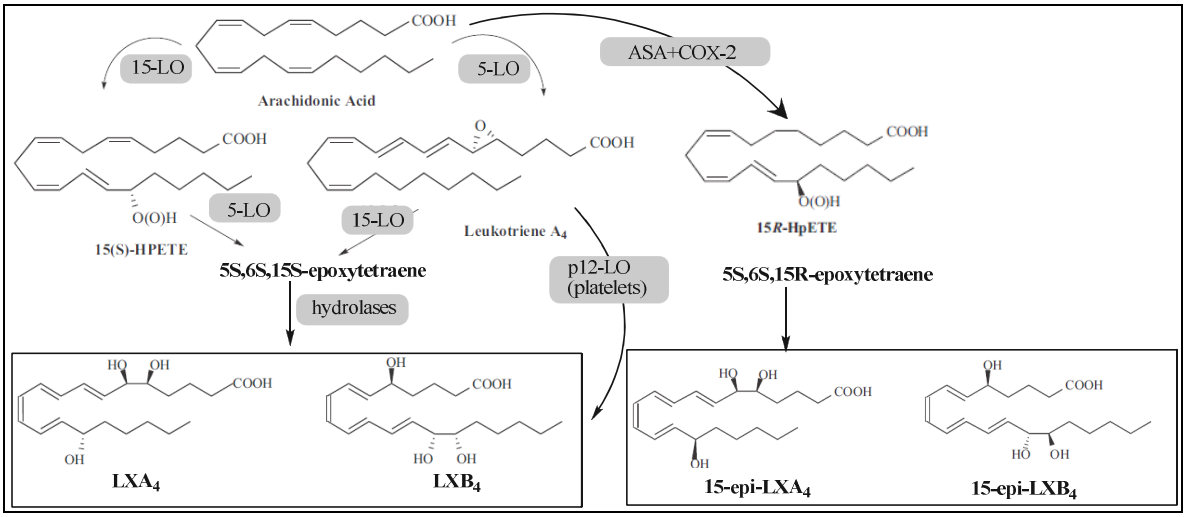


Fig. 5 Lipoxin biosynthesis [47], modified

15(S)-H(P)ETE derived from the oxidation of AA by 15-LO, is converted by 5-LO via an epoxytetraene to lipoxin A_4 (LXA_4) and lipoxin B_4 (LXB_4) . Another route involves LTA_4 from the 5-LO pathway which is converted by 15-LO or platelet-type-12-LO (p12-LO) by mean of cell-cell-interactions yielding LXA_4 and LXB_4 (**Fig. 5**).

An additional class of lipoxins is generated by acetylsalicylic acid (ASA)-treated COX-2 which acetylates the active site of COX-2. Now, AA is converted by the acetylated COX-2 to 15(R)-HETE. Then, 5-LO from PMNL further metabolizes 15(R)-HETE to 15-epi-LXs, the "aspirin-triggered-lipoxins" (ATL) [47] (**Fig. 5**). LXA_4 and ATL bind to a specific receptor, termed ALX or FPRL1, a GPCR belonging to the formyl peptide receptor family [48].

Beside AA, the ω-3 PUFAs EPA and DHA serve as precursors for resolvins, protectins and maresins, additional important mediators for the resolution of acute inflammation [5].

1.7 Leukotrienes

1.7.1 Leukotriene biosynthesis

LTs represent a unique family of eicosanoids derived from AA. The term implicates the presence of three conjugated double bonds within the 20-carbon structure [49]. Leukotriene biosynthesis is mediated by the action 5-LO and other enzymes (**Fig. 6**).

After release of AA from cellular membrane lipids by $cPLA_2$, molecular oxygen is inserted at C-5 yielding 5(S)-hydroperoxy-6,8-trans-11,14-cis-eicosatetraenoic acid (5-HPETE) followed by subsequent conversion to the unstable epoxide intermediate 5(S)-trans-5,6-oxido-7,9-trans-11,14-cis-eicosatetraenoic acid (LTA_4) [50]. A non-enzymatic hydrolysis of LTA_4 is observed *in vitro*, resulting in the formation of the diastereomeric dihydroxy derivatives 5(S),12(R)-diHETE (6-trans-LTB_4) and 5(S),12(S)-diHETE (6-trans-12-epi-LTB_4) [51].

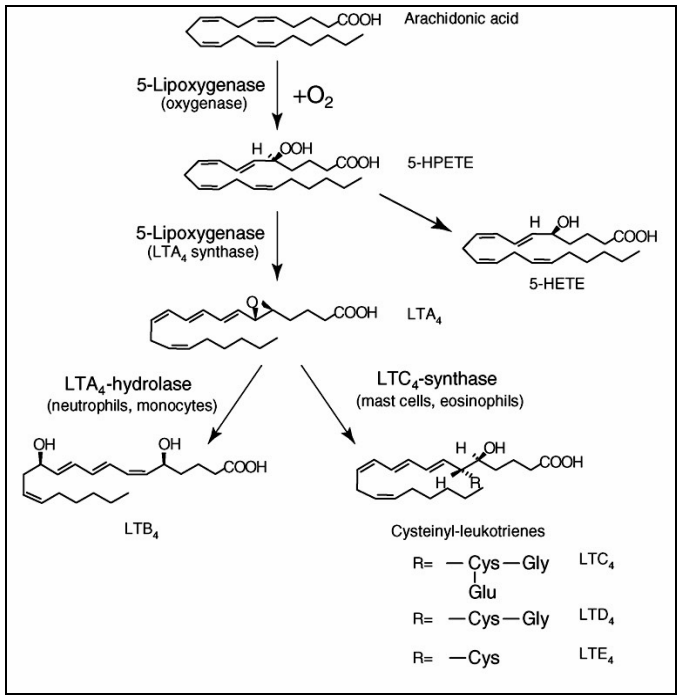


Fig. 6 Leukotriene biosynthesis [52]

5-HETE, the corresponding alcohol of 5-HPETE, can be metabolized by the microsomal enzyme 5-hydroxyeicosanoid dehydrogenase (5-HEDH). The resulting pro-inflammatory 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) is a strong chemoattractant for eosinophils [53]. In neutrophils, 5-oxo-ETE stimulates Ca^{2+} mobilization and cell migration and stimulates degranulation. It acts through the highly specific G_{α}-coupled OXE receptor, which is highly expressed on eosinophils and to a lower extent on neutrophils and macrophages. Moreover, prostate cancer cells contain high levels of 5-HEDH and an enhanced 5-oxo-ETE synthesis was found in apoptotic cells, suggesting a role for 5-oxo-ETE in cancer development [54].

LTA_4 is further converted enzymatically by LTA_4-hydrolase (LTA4H) to LTB_4 (5(S),12(R)-dihydroxy-6,8,10,14-eicosatetraenoic acid). LTA4H is a monomeric enzyme with a zinc-binding motif in the catalytic site and has a molecular weight of approximately 70 kDa [55]. LTA4H was isolated first from the soluble fraction of human leukocyte homogenates [56]. The conversion of LTA_4 to LTB_4 by LTA4H involves the opening of the epoxide at C-5 and the nucleophilic addition of water at C-12 with the participation of Glu271, Asp375

and the catalytic zinc ion. Beside its epoxide hydrolase function, LTA4H possesses an aminopeptidase activity, which seems to be involved in peptide reactions related to host defense and inflammation [57].

LTB_4 and 5-HETE were identified in the early 1980s when PMNL from rabbit [58] or human origin were incubated with AA and calcium ionophore A23187 [59]. LTB_4 is a very potent chemotactic molecule and stimulator of various leukocyte functions [60]. Over the years, an important pathophysiological role in asthma, allergic diseases [61], [62], cardiovascular and inflammatory conditions was attributed to LTB_4 [60].

Alternatively, LTA_4 is conjugated with GSH leading to the cysteinyl-LT LTC_4, catalysed by LTC_4 synthase (LTC4S), an 18-kDa, membrane-associated enzyme. For the reaction of LTA_4 to LTC_4, LTC4S binds GSH in its active site. LTC4S belongs to the MAPEG (membrane-associated protein in eicosanoid and glutathione metabolism) protein family along with the homologous mPGES-1 [63], 5-lipoxygenase-activating protein (FLAP) and microsomal glutathione S-transferases (MGST) [64].

After release of LTC_4 from the cell, peptide cleavage by γ-glutamyl transpeptidase and dipeptidases lead to the other cysteinyl-LTs (cys-LTs) LTD_4 and LTE_4, respectively [49]. LTC_4, LTD_4 and LTE_4 were first referred to as "slow-reacting substances of anaphylaxis" (SRS-A), underlining the role of cysteinyl-LTs in allergic conditions [65]. Cys-LTs cause bronchoconstriction in airway smooth muscle cells, increase microvascular permeability, reduce myocardial contractility and coronary blood flow [50]. They are the most potent bronchoconstricting mediators in humans and exert pro-inflammatory effects. Cys-LTs are implicated in cancer, cardiovasular disorders such as myocardial infarction and unstable angina as well as in atopic dermatitis and rheumatoid arthritis [66].

Additionally, LT biosynthesis occurs via transcellular mechanisms, where LTA_4 is transported and uptaken by other cells devoid of 5-LO. Thus, platelets

expressing LTC4S, but no LTA4H, take up LTA_4 from 5-LO expressing cells and produce LTC_4. In contrast, erythrocytes express no 5-LO, but LTA4H and convert the incorporated LTA_4 to LTB_4 [67].

1.7.2 Leukotriene receptors

LTs and Cys-LTs mediate their effects through cell-surface GPCRs. LTB_4 binds with high affinity to the specific BLT1 receptor, which was cloned in 1997 [68]. BLT1 is highly expressed on peripheral blood leukocytes and mast cells [69] and with lower incidence in spleen and thymus. Binding of LTB_4 to BLT1 mediates leukocyte functions such as chemotaxis or production of ROS [70]. In contrast, the BLT2 receptor subtype is more ubiquitously expressed with about 20-fold lower affinity for LTB_4 and different pharmacological properties compared to BLT1 [71]. BLT2 binds also other eicosanoids such as 12-HHT, 12-HETE and 15-HETE [72]. A recent study with BLT2-knockout mice suggested a role in inflammatory arthritis [73]. Another study observed an elevated BLT2 expression in bladder cancer [74]. However, a clear functional role for the BLT2 receptor subtype was not clarified yet.

Additionally, LTB_4 binds to the nuclear transcription factor PPARα, suggesting further anti-inflammatory functions [75]. Due to the important pathophysiologial roles of LTB_4, multiple selective and non-selective antagonists at the BLT receptors have been developed and studied for the treatment of rheumatoid arthritis, COPD, cystic fibrosis, osteoporosis as well as different types of or cancer, but no substance entered the market so far [76].

For the cys-LTs, two main receptor subtypes ($CysLT_1$ and $CysLT_2$) were characterized. The $CysLT_1$ receptor binds LTD_4 with high affinity, followed by LTC_4 and LTE_4 with less affinity. In contrast, the $CysLT_2$ subtype has equal affinity for LTC_4 and LTD_4 and lower affinity for LTE_4 [77]. The $CysLT_1$ receptor is expressed in blood cells such as eosinophils, monocytes, macrophages, neutrophils, in a subset of B-lymphocytes and mast cells and to a lesser extent in spleen, lung and smooth muscle cells. A significant increased

number of cells expressing the $CysLT_1$ receptor in patients with stable or exacerbated asthma supports the role of cys-LTs in inflammatory airway diseases. In contrast, the $CysLT_2$ receptor subtype is highly expressed in the heart and coronary arterial smooth muscle cells, in the brain, spinal cord and adrenal glands. Interestingly, the $CysLT_2$ receptor was also found in eosinophils and mast cells in patients with allergic rhinitis, but the role was undefined yet [66],[78].

In the meantime, additional receptor subtypes for cys-LTs were reported. The phylogenetically related GPR17 receptor, a dual CysLT and nucleotide receptor, binds LTC_4 and LTD_4 in the nanomolar range [79]. In double-knockout mice deficient of both $CysLT_1$ and $CysLT_2$ receptors, a GPCR with preference for LTE_4 was discovered [80].

Pharmacological influence on the action of cys-LTs was achieved with the development of specific antagonists for the $CysLT_1$ receptor. Montelukast (Singulair ®), zafirlukast (Accolate ®) and pranlukast (Onon ®) are approved and clinically used in the treatment of asthma and allergic rhinitis. BAYu9773 acts as dual antagonist at both receptor subtypes and shows partial agonism at the $CysLT_2$ receptor and is used only for experimental purposes [81]. Hence, the development of a selective $CysLT_2$ receptor antagonist would be helpful for further characterization of the receptor function. Recently, the first selective $CysLT_2$ receptor antagonist was described [82].

1.8 5-Lipoxygenase (5-LO)

1.8.1 Enzymatic reaction, structure and expression of 5-LO

5-LO was first isolated from homogenates of human leukocytes [83] and catalyzes the first steps in the biosynthesis of LTs (for details, see **Fig. 6**). 5-LO mediates the incorporation of molecular oxygen into AA (oxygenase activity) followed by subsequent dehydration of the resulting 5-HPETE to the allylic epoxide LTA_4 (LTA_4 synthase activity). LTA_4 serves as intermediate for the biosynthesis of LTB_4, cys-LTs or lipoxins [84]. The oxygenase reaction of 5-LO

is initiated by the abstraction of pro-S hydrogen at C-7 of AA [51]. In the next step, molecular oxygen is inserted at C-5, leading to 5-HPETE. The subsequent conversion to LTA_4 involves the abstraction of hydrogen at C-10 followed by allylic shifts to C-6 resulting in the formation of the 5,6-epoxide LTA_4 [85]. These dual enzymatic properties of 5-LO were first observed in purified enzyme from potato tubers [86] and confirmed in leukocyte homogenates [87] and murine mast cells [88].

5-LO is a monomeric enzyme with 673 amino acids, consisting of a large catalytical C-terminal domain (amino residues 121-673), whereas the N-terminal domain (1-114) is a Ca^{2+}-binding C2 domain [89] with similarity to other proteins such as $cPLA_2$ [90]. The residues in position 43-46 are involved in Ca^{2+} binding and three tryptophane residues (Trp75, Trp102 and Trp13) interact with phosphatidylcholine (PC), glycerides and coactosin-like protein (CLP) (**Fig. 7**).

The catalytic centre of 5-LO contains a non-heme iron atom, surrounded by His372, His550 and the C-terminal Ile673 as permanent ligands [91] forming a 2-His-1-carboxylate facial triad, a common structural LO motif. Asn554 and His367 were identified as replaceable ligands and H_2O was assumed as sixth ligand for the iron [92]. The catalytic domain of 5-LO contains three motifs for phosphorylation by the kinases ERK2, MK2 and PKA (**Fig. 7).**

For a long time, no crystal structure of 5-LO was available. Accordingly, all structural data were based on a homology model of rabbit reticulocyte 15-LO [43] or soybean-lipoxygenase-1 [93]. Recently, the crystal structure of 5-LO was presented at 2.4 Å resolution. Replacement of the Lys653-Lys655 sequence in the C-terminal region of 5-LO by the corresponding sequence of a 8R-LO enabled the stabilization of the enzyme for crystallization [94].

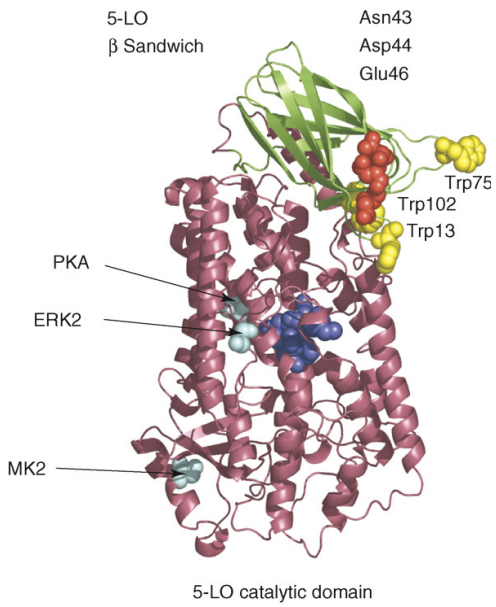


Fig. 7 Structure of 5-LO [52]
The N-terminal domain (residues 1–114; green) contains the Ca^{2+}-binding C2 domain. Mutagenesis of the amino acids 43–46 (red) reduced Ca^{2+} binding to 5-LO. Trp13, Trp74 and Trp102 (yellow) mediate the effects of PC, glycerides and coactosin-like protein (CLP). Phosphorylated serine residues in the kinase motifs for MK2, ERK2 and PKA are shown in light blue.

5-LO expression is mainly found in cells from myeloid origin such as leukocytes, monocytes, macrophages, mast cells, B-lymphocytes or dendritic cells [95]. Furthermore, 5-LO expression was reported in pulmonary endothelial cells [96] and arterial walls of atherosclerotic lesions [97]. For *in vitro* studies, 5-LO expression can be induced by differentiation of promyelocytic HL-60 cells with DMSO [98]. Moreover, addition of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2 D_3$) together with TGF-β [99] upregulates the enzymatic activity in the DMSO-treated HL-60 cells as well as in monocytic Mono Mac 6 cells [100].

1.8.2 The 5-LO gene

The human 5-LO gene (ALOX5) contains 14 exons, divided by 13 introns, and has a length of approximately 82 kb. In contrast to all other lipoxygenases. ALOX5 is located on chromosome 10. The promoter region lacks TATA and CCAT sequences, resembling the structure of an ubiquitously expressed housekeeping gene [101]. In total, 10 GC-rich regions (GC boxes) are present in the sequence, whereof 8 GC boxes are located in the promoter region [102]. 5

GC boxes in the promoter region are arranged in tandem [101] and were identified as binding sites for the transcriptional factors Egr-1 and Sp-1 [103]. Additional putative binding sites for transcription factors such as NFκB and AP-2 were found in the 5-LO promoter [104]. Naturally occurring mutations in the tandem GC boxes of the 5-LO promoter result in the deletion or addition of Sp-1/Egr binding sites. These variations were shown to influence the transcription of 5-LO [105]. Moreover, an association between different promoter genotypes and a diminished response to an oral 5-LO inhibitor in asthmatic patients was reported [106]. Carriers of polymorphisms in the tandem GC boxes in the 5-LO promoter showed an increased carotid intima-media-thickness, a marker for atherosclerosis and significantly higher risk of atherosclerosis [107]. Variants of ALOX5 were associated to tuberculosis susceptibility, underlining the role of LTs in the defense of bacterial infections [108]. Furthermore, interactions of retinoid receptors (RXR) and vitamin D receptor (VDR) to vitamin D responsive elements (VDRE) were reported in the 5-LO promoter region. However, these sites are not responsible for the induction of 5-LO expression by $1,25(OH)_2D_3$ and TGFβ [109]. 5-LO gene expression is regulated by DNA methylation and histone acetylation. Demethylation of the promoter is required for transcription of the 5-LO gene [110], whereas inhibition of histone deacetylation by trichostatin A strongly enhances 5-LO promoter activity and increases gene expression [111]. Recently, several spliced isoforms of 5-LO where identified in leukocytes. Due to lack of amino acid sequences in the catalytic domain, a decreased product formation and a different cellular distribution compared to full length 5-LO was observed [112].

1.8.3 Factors regulating 5-LO activity in the cell and *in vitro*

5-LO activity in the cell and *in vitro* is regulated in a complex manner involving several pathways and factors. One critical parameter is the availability of the substrate AA for 5-LO. In the cell, $cPLA_2$ liberates AA from membrane phospholipids upon stimulation. Thus, stimulation of cells with naturally ligands such as fMLP, C5a or PAF results in a low LT synthesis *in vitro*, related to low

substrate supply. 5-LO product formation can occur also independently of $cPLA_2$, since addition of exogenous AA strongly enhances 5-LO activity [113]. Over time, a variety of studies revealed that ATP, interaction with Ca^{2+} and PC, the intracellular peroxide tone, phosphorylation by kinases and the subcellular localization of the enzyme are important factors that control the enzymatic activity of 5-LO *in vitro*.

1.8.3.1 ATP

Experiments with peritoneal PMNL from guinea pigs showed that the activity of 5-LO is stimulated by ATP. Other nucleotides such as ADP, AMP, cAMP, GTP or CTP showed weaker or no effects on the enzyme activity [114]. Other studies confirmed that this effect requires Ca^{2+} [83], [115]. Interestingly, hydrolysis of ATP to ADP is not necessary for the upregulation of 5-LO product formation. Affinity chromatography with ATP is used for the purification of recombinant 5-LO. This fact raised the question for an ATP-binding site at the enzyme. Photoaffinity labeling studies with radioactive marked ATP analogues revealed a general nucleotide binding site, but not specific for ATP in the 5-LO sequence [116]. However, binding of ATP binding is characteristic for 5-LO, since other LOs do not have nucleotide binding sequences and seems to be important for the stabilization of the 5-LO structure.

1.8.3.2 Ca^{2+}, phospholipids and the C2 domain of 5-LO

The observation that stimulation of human PMNL with A23187 results in strong formation of 5-HETE and LTB_4 [59] substantiates the important role of Ca^{2+} for 5-LO activity. For isolated recombinant 5-LO, the required concentration for half-maximal enzyme activity is about 1-2 µM, whereas for full activation 4-10 µM is needed [117]. In contrast, much lower levels of intracellular Ca^{2+} of 350-400 nM are necessary for cellular 5-LO product formation. Thus, Ca^{2+} release from intracellular stores is sufficient for 5-LO activation in PMNL independently from $cPLA_2$ activation [118].

5-LO binds two Ca^{2+} ions per molecule at its N-terminal domain with an K_d of 6 µM thereby increasing the hydrophobicity of the enzyme [119]. The N-terminal β-barrel domain of 5-LO shares high similarity to Ca^{2+}-binding C2 domains of other proteins such as PKC or $cPLA_2$. Mutagenesis studies identified Asn43, Asp44 and Glu46 (**Fig. 7**) as putative Ca^{2+} ligands of 5-LO [89]. Ca^{2+} improves the binding of PC to 5-LO and thereby promotes the conversion of 5-HPETE to LTA_4 [120]. Three surface-exposed tryptophane residues (Trp13, Trp75 and Trp102) in the C2 domain bind PC and mediate the translocation of 5-LO to membranes [121],[122] (**Fig. 7**). However, when high amounts of PC are present, Ca^{2+} is not strictly required for 5-LO activity [123]. Furthermore, binding of Ca^{2+} to the C2 domain protects the enzyme from the inhibitory effects of GPx-1 and leads to 5-LO activation at lower peroxide levels [124].

Mg^{2+} can substitute for Ca^{2+} in 5-LO activation steps, but for maximal activity, a high concentration of 4 mM Mg^{2+} is needed. Activation by Mg^{2+} was also dependent on phospholipid and AA concentrations [125]. Beside Mg^{2+}, Mn^{2+}, Sr^{2+} and Ba^{2+} ions can activate 5-LO *in vitro*, but with much weaker effects than Ca^{2+} [117].

Interestingly, cellular 5-LO activation by chemical stress (sodium arsenite), osmotic stress (NaCl) or heat shock was independent of intra- and extracellular Ca^{2+} [126], showing that 5-LO activity is also regulated by Ca^{2+}-independent mechanisms.

1.8.3.3 Glycerides

The diacylglycerol 1-oleyl-2-acetylglycerol (OAG) strongly stimulates 5-LO product formation *in vitro* in a calcium-independent manner [127]. OAG binds to three tryptophan residues (Trp13, Trp75 and Trp102) at the C2 domain of 5-LO which serves also as binding site for phospholipids. Thus, the stimulatory effect of OAG is reversed by phospholipids. Other structural related glycerides showed weaker or no stimulatory effects on 5-LO product formation. Moreover, OAG increases the affinity of 5-LO for activating lipid hydroperoxides (LOOH)

necessary for catalysis and renders 5-LO activity resistant against GPx activity [128]. Additionally, the endogenous generation of diacylglycerides (DAG) resulting from hydrolysis of phosphatidylcholine mediated by phospholipase D (PLD) and phosphatidic acid phosphatase (PA-P) is a determinant for 5-LO product formation and 5-LO translocation to the nuclear membrane in PMNL [129]. Interestingly, gender-dependent PLD activity was recently reported resulting in higher 5-LO product formation in monocytes from female donors compared to cells from male donors. The suppressive effect on LT formation in male cells is caused by the sex hormone 5α-DHT resulting in phosphorylation of ERK2 and thus diminished DAG formation by PLD [130]. These findings exhibit DAGs as important determinants for the regulation of 5-LO activity in intact cells and *in vitro*.

1.8.3.4 Coactosin-like protein (CLP)

Investigations of protein interactions of 5-LO identified CLP, an F-actin binding 16-kDa protein with high homology to coactosin from *Dictyostelium discoideum* [131]. The stoichometry of CLP binding to 5-LO is 1:1 and mutation of Lys131 reduced the complex between 5-LO and CLP. Interestingly, the interaction between 5-LO and CLP is a calcium-independent process [132]. Additionally, Trp102 of 5-LO was found to mediate binding between 5-LO and CLP, which prevents non-turnover activation of 5-LO and stabilizes the 5-LO structure in the cell [133].

In the presence of PC, CLP increases the Ca^{2+}-induced 5-LO activity, promotes the formation of LTA_4 and increases the ratio of 5-HETE/5-HPETE formation *in vitro* [134]. The subcellular distribution of CLP correlates with 5-LO. Upon cell activation, 5-LO and CLP comigrate to the nuclear fraction in contrast to a cytosolic localization in the resting state [133]. In neutrophils, the co-migration of 5-LO and CLP was observed together with a gender-related distribution pattern in cells from male and female donors [135].

1.8.3.5 Lipid hydroperoxides (LOOH) and glutathione peroxidases (GPx)

For 5-LO catalysis, a threshold level of LOOH is necessary for the oxidation of the catalytic iron of 5-LO from the inactive ferrous (Fe^{2+}) to the active ferric (Fe^{3+}) state [136]. Selenium-dependent GPx regulate the hydroperoxide tone in leukocytes by reduction of hydroperoxides such as 5-HPETE and thereby control 5-LO activity [137]. In the resting cell, intracellular peroxide concentrations are kept low by the activity of GPx which reduce LOOH. Conditions that lead to oxidative stress or depletion of GSH *in vitro* increase the peroxide concentration, resulting in activation of 5-LO [138]. An elevated peroxide concentration strongly impaired the efficacy of the non-redox-type inhibitors ZM230487 and L-739,010 [139].

1.8.3.6 5-LO phosphorylation by kinases

Proinflammatory stimuli, cell stress or heat shock lead to activation of p38 MAPK in PMNL and increased LT synthesis. Accordingly, p38 MAPK activates the downstream kinases MK2/3, which phosphorylate 5-LO *in vitro* [140]. Mutation experiments revealed Ser271 as phosphorylation site and AA and other unsaturated fatty acids strongly enhance the phosphorylation state of 5-LO. In contrast, the phosphorylation of 5-LO by CaMKII and the catalytic subunit of protein kinase A (PKA) was found to occur independently of exogenous AA addition [141].

Furthermore, extracellular signal-regulated kinases (ERKs) regulate 5-LO activity. ERK2 phosphorylates 5-LO at Ser663 and as shown for MK2, addition of AA and other unsaturated fatty acids strongly enhanced the effect *in vitro.* The phosphorylation state correlated with the amount of product formation, suggesting the kinase actions as an important factor for cellular 5-LO activity. Product formation in PMNL induced by the physiological agonist fMLP or AA was reduced by the MEK inhibitor U0126, confirming the involvement of ERK2 in this process [142]. Thus, AA-induced product formation in PMNL leads to phosphorylation of 5-LO at different sites by ERK2 and p38 MAPK-regulated MK2. Interestingly, AA-induced product formation in PMNL occurs

independently of Ca^{2+}, suggesting alternative mechanisms of 5-LO activation in the cell [143].

Recently, a gender-dependent activation status of ERK was shown to cause different LT formation and the subcellular distribution of 5-LO in PMNL from male and female donors. The androgen 5α-DHT causes activation of ERK, resulting in lower 5-LO product formation in male cells [135]. In contrast, phosphorylation of 5-LO by PKA at Ser523 attenuates LT biosynthesis [144] and impairs the nuclear import of 5-LO [145]. An elevation of intracellular cAMP levels activates PKA and prevents 5-LO translocation and subsequently LT formation [146]. To sum up, phosphorylation of 5-LO by p38 MAPK-regulated MKs, ERK and PKA regulates the catalytic activity of 5-LO in intact cells in a Ca^{2+}-dependent manner.

1.8.4 5-lipoxygenase-activating protein (FLAP)

The integral membrane-bound, 18-kDa protein FLAP plays a substantial role for cellular LT biosynthesis [147]. Investigations with the leukotriene synthesis inhibitor MK-886 (using a radiolabelled derivative thereof) led to the identification of FLAP in rat neutrophil extracts [148]. The high relevance of concurrent FLAP and 5-LO expression for LT formation was shown in transfection experiments with osteosarcoma cell lines [147] and differentiated HL-60 cells [149]. FLAP directly binds AA and transfers it to 5-LO thereby affecting the ratio of formed 5-HPETE and LTA_4 [150], [151]. The specific binding of AA to FLAP is competed by inhibitors such as MK-886 [150]. FLAP belongs to MAPEG family. In contrast to the other five human MAPEG members such as mPGES-1 or LTC4S, it has no enzymatic activity and its function is not modulated by GSH [152]. The three-dimensional structure of FLAP was solved in 2007 and shows a homotrimer, whereof each monomer consists of four transmembrane helices connected by two cytosolic and one lumenal loop [153] (**Fig. 8**).

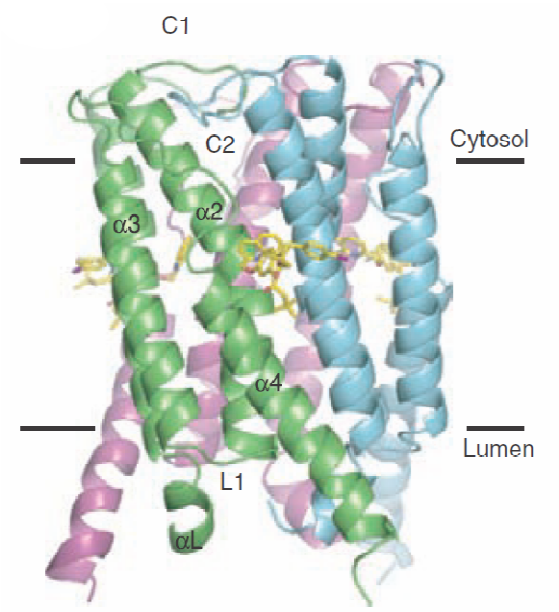


Fig. 8 Structure of FLAP in complex with inhibitor MK-591 [153]
The three FLAP monomers are colored in green, blue and magenta. The bound inhibitor MK-591 is presented as stick model in yellow. C1 and C2 designate the cytosolic, L1 the luminal connecting loop of the FLAP homotrimer.

The expression of FLAP has a pro-inflammatory and pathophysiological role and was linked to atherosclerosis, metabolic diseases and obesity [154]. Polymorphisms in the FLAP gene (ALOX5AP) were associated with higher risk of myocardial infarction and stroke due to enhanced LT formation and a higher inflammatory state [155]. However, a meta-analysis of studies investigating ALOX5AP gene polymorphisms doubted a significant correlation [156]. Nonetheless, FLAP is a specific, promising target for the development of potent LT biosynthesis inhibitors for the treatment of inflammatory and cardiovascular diseases.

1.8.5 Subcellular distribution of 5-LO

Early studies indicated that 5-LO is a mobile enzyme in the cell. The initial step of LT biosynthesis requires the calcium-dependent movement of 5-LO from the cytosol to a membrane compartment [157]. This translocation process was observed in osteosarcoma cells [158], HL-60 cells [159] and human leukocytes [160] after stimulation with Ca^{2+}-ionophore A23187 or the chemoattractant fMLP, where 5-LO was detected in the membrane fraction after cell disruption.

Binding of Ca^{2+} to the N-terminal C2-domain increases the hydrophobicity of 5-LO and promotes translocation to the nuclear membrane [119], [121]. The nuclear membrane is referred to as "metabolon", since it is the site where LT synthesis takes place [161]. Upon elevation of intracellular calcium, both 5-LO and $cPLA_2$ move from the cytosol to the nuclear membrane, where $cPLA_2$ liberates AA from membrane phospholipids [162]. Then, the integral membrane protein FLAP binds AA and transfers it to 5-LO where the catalytic reaction generates 5-HPETE and LTA_4 [163] **(Fig. 9)**.

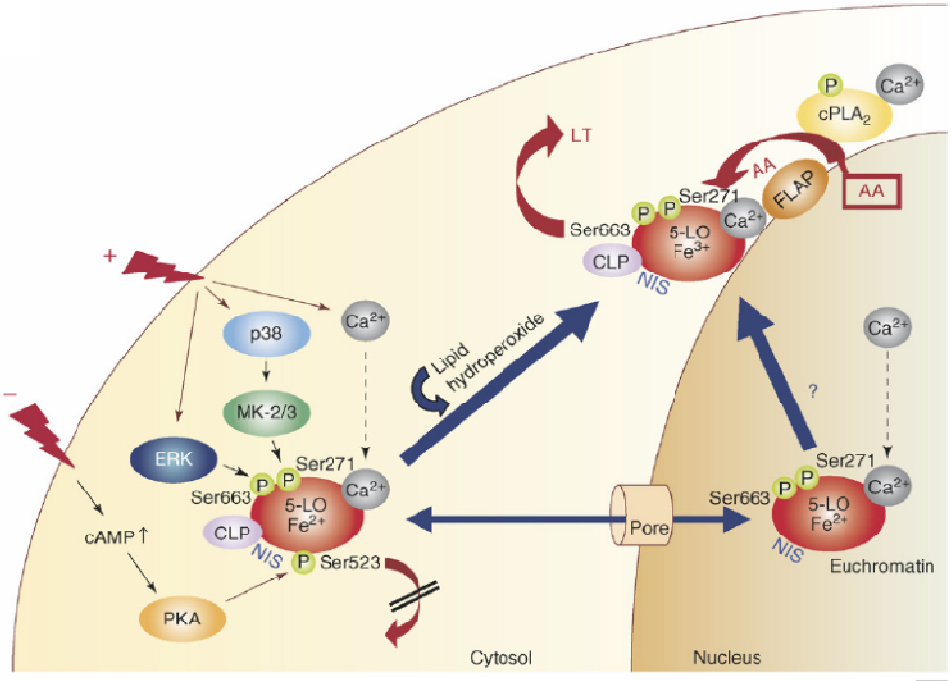


Fig. 9 Intracellular 5-LO distribution ([52], modified)
In the resting state, 5-LO is a cytosolic enzyme. 5-LO catalysis is activated by calcium or phosphorylation by ERK and p38 MAPK. Lipid hydroperoxides convert Fe^{2+} in the catalytic site to $Fe^{3+}$and 5-LO translocates to the nuclear membrane where it colocalizes with $cPLA_2$, CLP and FLAP followed by conversion of AA to 5-HETE and LTs. abbreviations: NIS: nuclear import sequence

LTA_4 serves as substrate for the integral membrane protein LTC_4 synthase yielding LTC_4 [63] or for the conversion to LTB_4 by leukotriene A_4 hydrolase. The ratio of generated LTC_4 and LTB_4 depends on the formation of different heteromers of FLAP and LTC_4 synthase at the nuclear membrane [164]. The FLAP inhibitor MK-886 [165] and other indole- and quinoline-based

compounds [159] can inhibit and reverse the membrane association of 5-LO and thus reduce LT formation in the cell. The intracellular localization of 5-LO depends on the cell-type. 5-LO is located in the cytosol of resting neutrophils, monocytes and peritoneal macrophages [163]. In contrast, in resting human alveolar macrophages [166] and rat basophilic leukemia cells [167], 5-LO was present predominantly in the nucleus. The nuclear 5-LO showed enzymatic activity and also translocates to the nuclear envelope after cell activation. Adherence of neutrophils or recruitment to inflammatory sites caused a nuclear import of 5-LO and increased LT formation [168]. Nuclear localization sequences (NLS) at the residues 638-655 and two nuclear export sequences (NES) control the nuclear localization of 5-LO [169]. A nuclear localization of 5-LO influences the capacity for LTB_4 formation in stimulated cells [170]. 5-LO phosphorylation at Ser271 by p38 MAPK-regulated MKs prevents nuclear export of 5-LO and increases LTB_4 formation [171]. In contrast, phosphorylation of 5-LO at Ser523 by PKA suppresses 5-LO activity by prevention of nuclear import [145].

Interestingly, 5-LO distribution in human neutrophils was found to be gender-dependent. In resting male neutrophils, 5-LO was found in the cytosol as well as in the nuclear memrane fraction regardless of cell stimulation, resulting in lower LT formation in activated male cells. In contrast, in neutrophils from female donors, a clear 5-LO translocation from the cytosol to the nuclear membrane in response to A23187 or LPS/fMLP was observed. The observed gender-dependent differences are related to the activation state of ERK regulated by the androgen 5α-DHT [135].

1.9 Pathophysiological implication of the 5-LO pathway

The conversion of AA by 5-LO and the associated enzymes for LT biosynthesis yields lipid mediators with central importance in inflammatory, allergic and cardiovascular processes, the innate immune system and cancer. Studies with knockout mice confirmed the pathophysiological roles of the 5-LO pathway. Thus, it was observed that 5-LO-deficient mice showed a resistance to PAF-

induced lethal shock and reduced inflammatory response to exogenous AA, underlining the proinflammatory properties of 5-LO-derived mediators [172]. Furthermore, 5-LO knockout mice showed an impaired bacterial phagocytosis and killing and compared to wild-type mice, a higher lethality in Klebsiella pneumoniae infection was observed [173]. Generally, LTs amplify killing of microorganisms and enhance phagocytosis of macrophages and neutrophils [174]. Thus, LT-deficient mice showed impaired immune responses in chronic fungal infection [175]. These results point out a participation of LTs in the host defense of infections and other immune responses.

LTs are known as bronchoconstrictory and vasoactive molecules contributing to asthmatic and allergic responses [60]. In patients with aspirin-sensitive asthma, atopic dermatitis and allergic rhinitis, LTB_4 levels are increased and thus treatment with BLT1 receptor antagonists or direct 5-LO inhibitors might be useful [61]. In particular, the contribution of Cys-LTs is clearly evident in allergic and asthmatic conditions due to their actions on the smooth muscle tone in the airways, microvascular permeability and their established pro-inflammatory role. In allergic rhinitis, the use of the $CysLT_1$ receptor antagonists zafirlukast, montelukast or pranlukast alone or in combination with antihistaminic drugs such as loratadine significantly reduced the symptoms [65].

Polymorphisms of the genes for FLAP (ALOX5P) and LTA_4 hydrolase or genetic variations in the 5-LO promoter were associated to higher risk for atherosclerosios, stroke and myocardial infarction [176], [177]. Also, a possible role of the 5-LO pathway in abdominal aortic aneurysm seems likely as shown in various mouse models [178]. Since the expression of 5-LO, associated enzymes of LT biosynthesis and LT receptors was detected in atherosclerotic lesions [97] and the 5-LO pathway is involved in various atherogenic processes [179], [180], anti-LT drugs may be useful in the treatment of cardiovascular diseases.

Moreover, expression of 5-LO, the associated enzymes for LT biosynthesis and LT receptors as well as elevated levels of LTB_4 were reported in a large number of cancer cells and tissues such as colon, oesophageal, lung, prostate, pancreatic, breast and skin cancer. Accordingly, inhibition of the 5-LO pathway decreased cell proliferation, inhibited angiogenesis and induced apoptosis. In addition, the 5-LO metabolites 5-HETE and LTB_4 induce cell proliferation and promote tumor growth in various cancer cells [181], [182], [16]. In addition, FLAP inhibition [183] or targeted gene disruption of 5-LO [184] modulated the activity of γ-secretase suggesting the 5-LO pathway as a target in Alzheimer's disease and other neurodegenerative disorders [185], [186].

1.10 Pharmacological inhibition of LT biosynthesis

The broad involvement of the leukotrienes in numerous pathophysiological conditions proposes therapeutic benefit of pharmacological intervention with specific components of the biosynthetic pathway. Since 5-LO is the key enzyme for the formation of 5-H(P)ETE and LTA_4 from AA, either a direct enzyme inhibition or the modulation of the catalytic activity of 5-LO is conceivable. In addition, other enzymes involved in LT biosynthesis such as LTA_4 hydrolase, LTC_4 synthase or FLAP are possible targets for specific inhibitors. For example, several LTA4H inhibitors were identified [187], [188] and designed by the use of a pharmacophore model [189]. Receptor antagonists at the LTB_4 and Cys-LT receptors on the target cells offer an alternative mechanism to prevent the biological action of LTs and Cys-LTs.

1.10.1 5-LO inhibitors

Inhibitors of 5-LO product formation either directly intervene with 5-LO or prevent the catalytic activity. Direct inhibitors of 5-LO are classified into redox-type inhibitors, iron-ligand or non-redox-type inhibitors according to their mode of action. A fourth class involves compounds with other or an unknown mode of action.

1.10.1.1 Redox-type-inhibitors

In particular, lipophilic molecules comprising polyphenolic (**Fig. 10**), quinone, coumarin or similar structures are often found in natural sources. These compounds reduce 5-LO product formation via redox-active mechanisms by keeping the active site iron of 5-LO in the ferrous (Fe^{2+}) state, reduce LOOHs or scavenge electrons and thus interrupt the catalytic cycle and prevent activation of 5-LO. *In vitro*, a very potent inhibition of 5-LO is often apparent in cellular and cell-free test systems. However, most of these compounds possess poor bioavailability and selectivity for 5-LO. Moreover, interference of these compounds with other biological redox systems leads to methemoglobin formation, production of ROS or other severe side-effects [190]. Despite further development of synthetic, orally active compounds such as AA-861 or BW-755C (**Fig. 10)** with weaker reducing properties, the aforementioned disadvantages of this class yielded no approved compound so far.

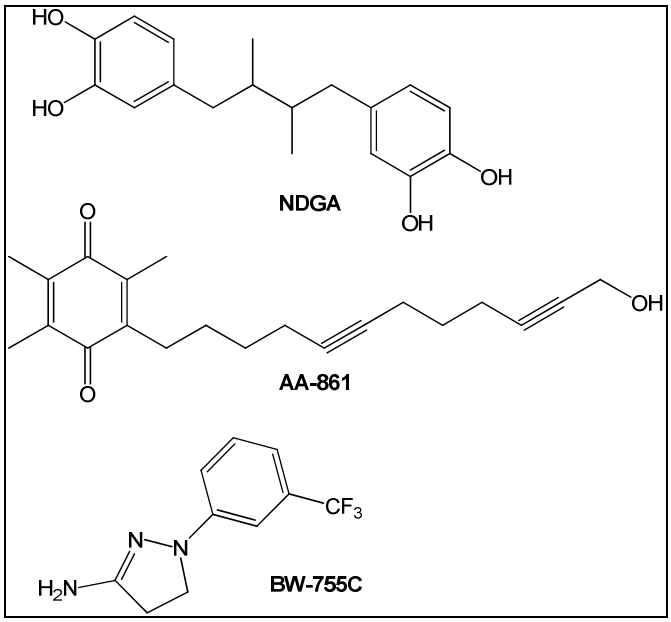


Fig. 10 Redox-type inhibitors of 5-LO

1.10.1.2 Iron ligand inhibitors

Iron ligand inhibitors chelate the active site iron of 5-LO and keep it in the ferrous state and in addition, exert weak reducing properties. Acetohydroxamic acid derivatives, exemplified by the compound BWA4C (**Fig. 11**) reduced LTB_4

formation very potent in intact human leukocytes and homogenates *in vitro* as well as *ex vivo* after oral administration [191]. However, the hydroxamate moiety is rapidly inactivated *in vivo* to the corresponding carboxylic acid [192].

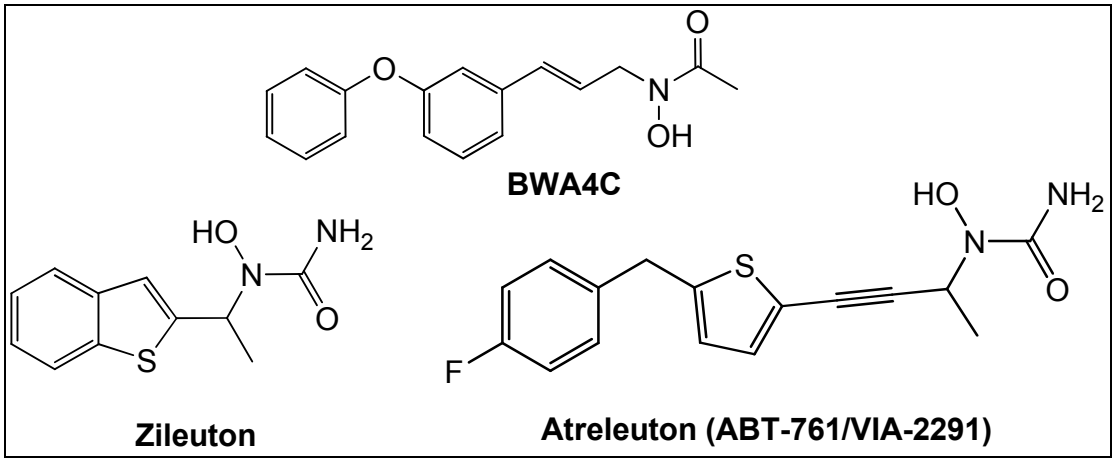


Fig. 11 Iron-ligand inhibitors of 5-LO

Beside their iron-chelating properties, the pseudoperoxidase activity of 5-LO contributes to the inhibitory mechanism of N-hydroxyurea and hydroxamate compounds leading to weak redox activity of the compounds towards 5-LO [193].

Structural optimization by introduction of an amino moiety led to the orally active N-hydroxy urea derivative zileuton (N-(1-benzo[b]thien-2-ylethyl)-N-hydroxyurea)) (**Fig. 11**). *In vitro,* zileuton reduced the biosynthesis of 5-LO-derived products in cell-based models and human whole blood with IC_{50} values < 1 µM and also *ex vivo,* a very potent inhibition of LTB_4 synthesis was apparent after peroral administration to rats and humans. Little or no inhbition of 12- or 15-LOs as well as COX enzymes was observed at concentrations up to 100 µM [194], [195]. Furthermore, the potent action of zileuton in asthmatic and other inflammatory airway and allergic diseases was confirmed in several clinical studies. Thus, zileuton was approved as direct 5-LO inhibitor on the market for the treatment of asthma. However, due to the short half-life of approximately 2.5 h, the four-times daily application and the elevation of hepatic enzymes limit the use of zileuton [196]. At present, an extended release

formulation for twice daily oral dosage is on the market in the USA (Zyflo CR), whereas the immediate release tablet was withdrawn in 2008. Recently, a use of zileuton for the treatment of acne was discussed [197], [198].

Atreleuton (ABT-761 or VIA-2291) was reported as a new candidate of iron-ligand inhibitors of 5-LO (**Fig. 11**). Atreleuton reduced the levels of LTs in patients with recent acute coronary syndrome in a placebo-controlled clinical trial with only low incidence of adverse events [199].

1.10.1.3 Non-redox-type inhibitors

A third class of 5-LO inhibitors is referred to as "non-redox-type inhibitors". These structurally diverse compounds (**Fig. 12**) are itself devoid of redox properties. Experimental data suggest a competition between activating LOOH and the compounds at a putative regulatory site of 5-LO.

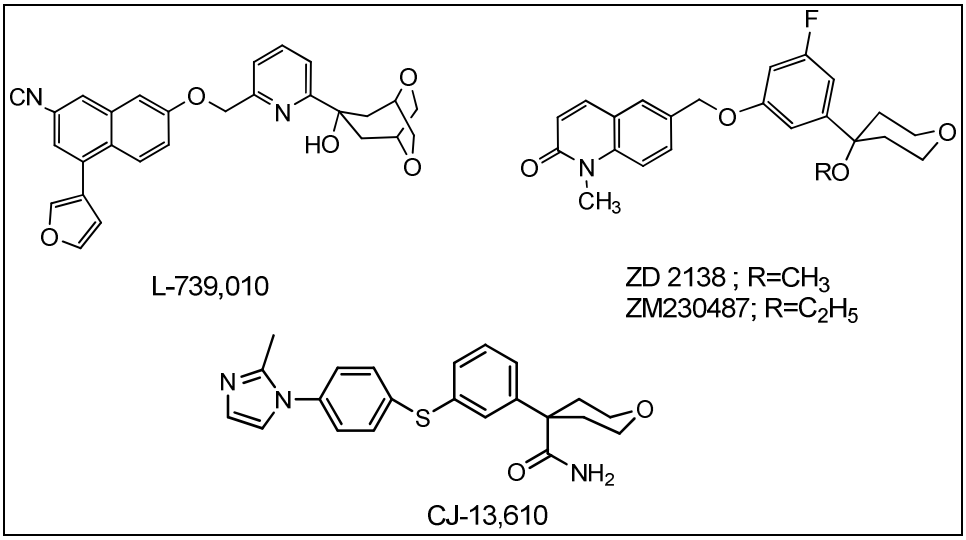


Fig. 12 Non-redox-type inhibitors of 5-LO

In vitro, ZM230487 and L-739,010 (**Fig. 12**) suppressed LT formation in granulocytes very potently in the nanomolar range, but in cell homogenates the potency of the compounds was strongly impaired. The addition of thiols such as GSH or DTT to the homogenates resulted in IC_{50} values comparable to intact cells. It was observed that the efficacy of the compounds depends on the peroxide tone in the cell, regulated by selenium-dependent GPx. Accordingly,

inhibition of GPx and elevated oxidative stress reduced the potency in cellular assays [139]. In addition, the efficacy of ZM234087 and L-739,010 was shown to be dependent on the activation pathway of 5-LO. Inhibition of cell-stress-induced 5-LO product formation required up to 100-fold higher concentrations compared to calcium-dependent activation of 5-LO by A23187 [200]. In contrast, the tetrahydropyrane-carboxamide CJ-13,610 (**Fig. 12**), inhibited 5-LO product formation indepently from the activation state of 5-LO activity in a competitive manner [201]. This fact shows an advantage of CJ-13,610 in the class of non-redox-type inhibitors, although the *in vivo* efficacy of all non-redox-type inhibitors is impaired in inflammatory conditions due to the elevated peroxide concentrations. Additional candidates of this class comprise coumarin derivatives developed by the Merck Frosst group with high anti-inflammatory efficacy and without toxic side-effects [202].

1.10.2 Novel-type inhibitors

Some compounds are described as inhibitors of 5-LO product formation, but cannot be classified among the abovementioned types of 5-LO inhibitors. Hyperforin, an acylphloroglucinol from St. John's Wort was identified as dual inhibitor of 5-LO and COX-1 with IC_{50} values in the low micromolar range [203]. Subsequent studies revealed that hyperforin interrupts the interaction of CLP and 5-LO and inhibits the translocation of 5-LO to the nuclear membrane, an early activation step of 5-LO regulation [204]. Moreover, boswellic acids (BA) from the gum resin of Boswellia serrata inhibit 5-LO product formation *in vitro* [205],[206]. Investigations about the mode of action of the acetyl-11-keto-BA (AKBA) suggested a binding of AKBA to a regulatory site of 5-LO [207]. These compounds demonstrate only two examples for several points of attack for the inhibition of 5-LO product formation.

1.10.3 Leukotriene receptor antagonists

The discovery of the LTB_4 receptors BLT1 and BLT2 and the cysteinyl-LT receptor subtypes $CysLT_1$ and $CysLT_2$ along with the involvement of LTB_4 and cysteinyl-LTs in various diseases suggest the use of receptor antagonists to

inhibit the actions of the lipid mediators. Accordingly, various compounds with affinity for the BLT receptors were described and investigated in clinical studies for COPD, rheumatoid arthritis, osteoporosis, psoriasis or cardiovascular diseases. Unfortunately, the selective blockade of the BLT receptors did not lead to sufficient efficacy. It was assumed that LTB_4 is not the only contributing mediator in some diseases [76].

A more promising approach is represented by the Cys-LT_1 receptor antagonists. Currently, montelukast, zafirlukast and pranlukast (**Fig. 13**) are approved nearly worldwide and used for the treatment of asthma, allergic rhinitis and urticaria. Especially in the treatment of asthma, the $CysLT_1$ receptor antagonists are considered as additional or alternative therapy to inhaled corticosteroids [81]. The pathophysiological role of the cys-LTs in other conditions such as endothelial dysfunction, cerebral and myocardial ischemia expand the field of application for these compounds [179].

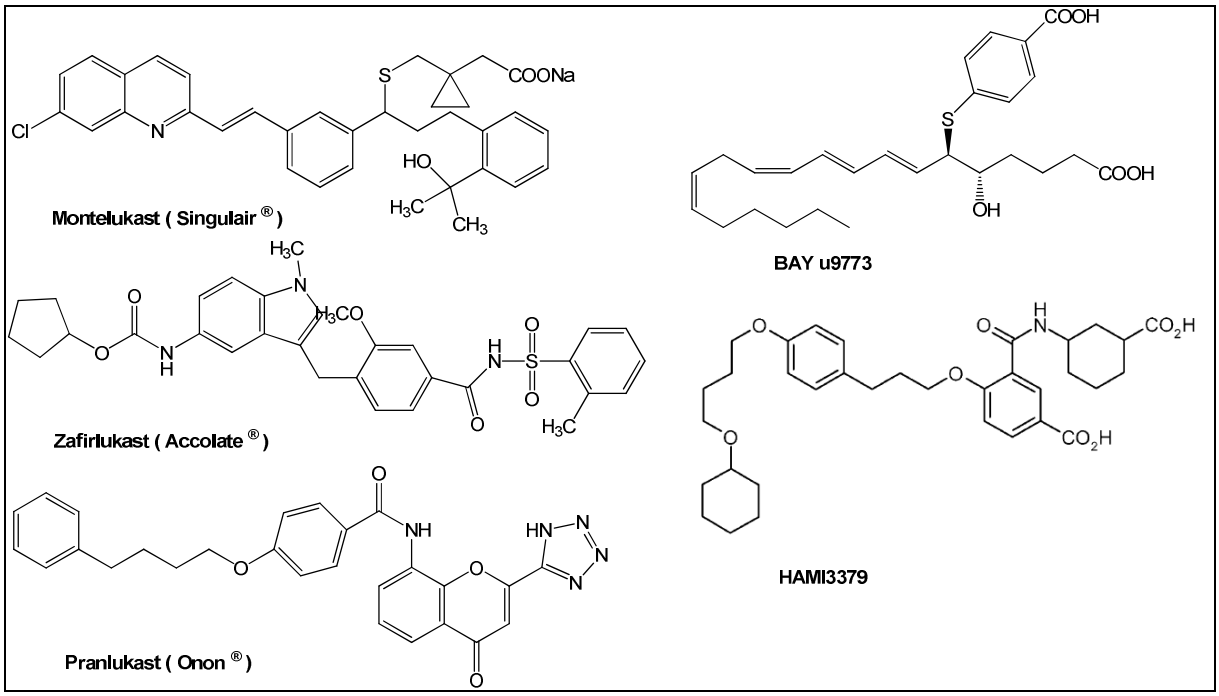


Fig. 13 Cys-LT receptor antagonists

In contrast, the number of reported $CysLT_2$ receptor antagonists is quite low. BAY u9773 (**Fig. 13**), an LTE_4 analogue was described as $CysLT_2$ antagonist,

but later characterized as dual antagonist at $CysLT_1$ and $CysLT_2$ receptor [208]. Recently, the potent and selective $CysLT_2$ antagonist, HAMI 3379 (**Fig. 13**) was reported [82].

1.10.4 FLAP inhibitors

The membrane-bound protein FLAP acts as a transfer protein for AA to 5-LO. Accordingly, an interference with the interaction between 5-LO and FLAP lead to total inhibition of 5-LO-derived products. The indole-based compound MK-886 (**Fig. 14**) was the first described inhibitor of FLAP with an IC_{50} of 2.5 nM in PMNL [209], followed by quinoline-based compounds [210]. Shortly afterwards, a novel class termed quindoles with a hybrid structure of indole and quinoline, represented by MK-591 (**Fig. 14**) was described [211], [212]. Photoaffinity labeling studies confirmed a direct binding of the indole, quinoline and quindole compounds to FLAP which results in the inhibition of LT biosynthesis.

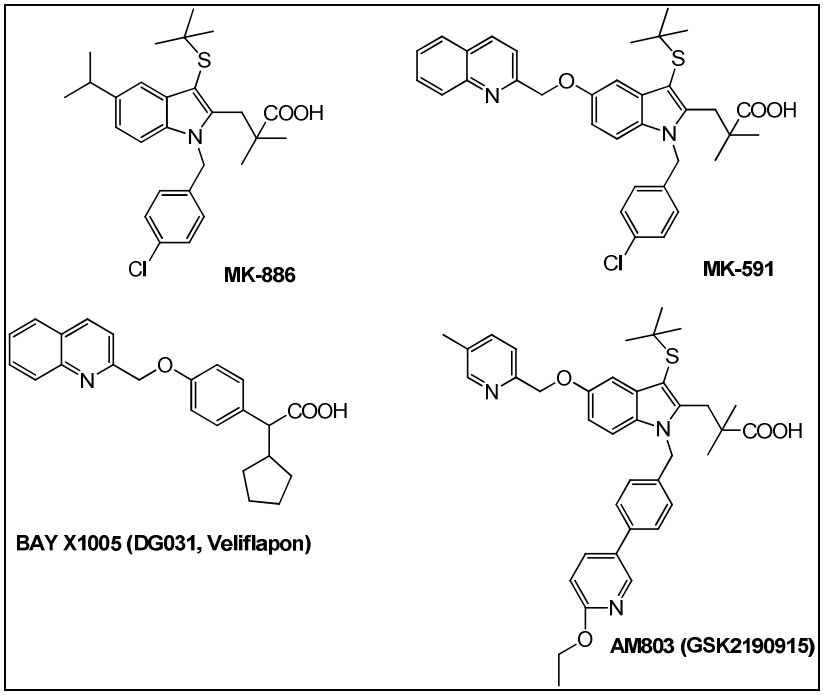


Fig. 14: FLAP inhibitors

FLAP inhibitors inhibit the cellular LT formation very potently whereas in cell-free assays no or only marginal influence of the compounds is evident.

Moreover, the FLAP inhbitor MK-886 was described to inhibit and reverse the translocation of 5-LO to the nuclear membrane thereby preventing the catalytic reaction of 5-LO [165]. The quinoline derivative BAYX1005 (now DG031, veliflapon) (**Fig. 14**) and MK-591 were investigated in clinical trials for asthma [152]. However, a clinical trial by deCode genetics for veliflapon in prevention of heart attack and stroke was suspended for unknown reasons [213].

Structural modifications based on the structures of MK-886 and MK-591 by Amira Pharmaceuticals led to a series of compounds such as AM103 [214] or the topical compound AM643 [215]. AM803 (**Fig. 14**), now designated as GSK2190915 [216] was investigated in various clinical studies in asthmatic indications [217].

1.10.5 The dual inhibition concept

The observation that inhibition of the COX pathway shifts AA metabolism towards the 5-LO pathway led to the idea of dual COX/5-LO inhibition [218]. A specific intervention with PG and LT biosynthesis offers a better anti-inflammatory effect and better gastrointestinal, renal and cardiovascular tolerability compared to NSAIDs [219], [220]. In addition, the co-expression of COX-2 and 5-LO in cancer and the effect of PGE_2 and 5-LO-derived eicosanoids in carcinogenesis may allow the use of dual COX/5-LO inhibitors as anti-cancer drugs [221]. Accordingly, several compounds were reported such as the pyrazole derivative tepoxalin (**Fig. 15).** Although a potent anti-inflammatory activity and low gastrointestinal toxicity was observed [222], the compound showed a broad inhibition of redox enzymes and liver toxicity [223], limiting the use in humans. Today, tepoxalin is used as anti-inflammatory drug in the veterinary medicine.

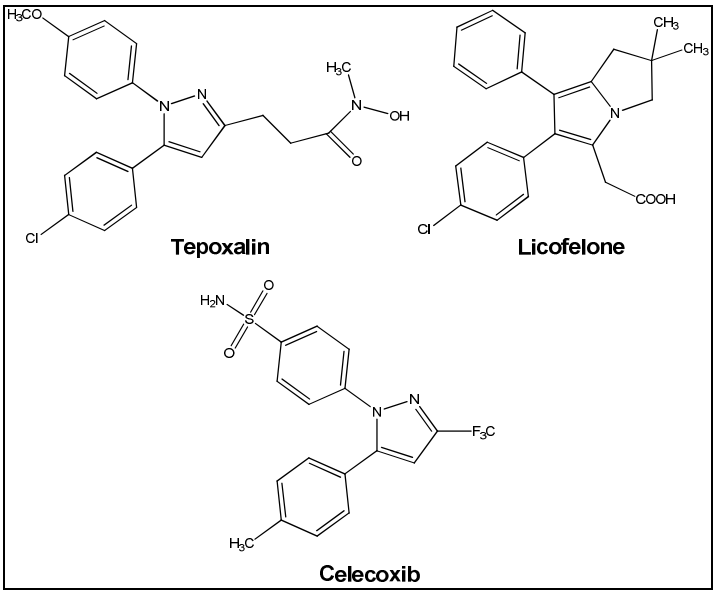


Fig. 15 Dual COX/5-LO inhibitors

Flavocoxid, a mixture of the flavonoids baicalein and catechin and marketed as "medical food" for the treatment of osteoarthritis, was described as dual inhibitor of COX and 5-LO with good anti-inflammatory properties *in vitro* [224], but caused acute liver injury [225].

Beside its known function as COX-2 inhibitor for the treatment of pain and arthritis, celecoxib (**Fig. 15)** inhibits 5-LO product formation in neutrophils as well as in human whole blood and reduced LTB_4 blood levels in rats [226]. Another promising dual COX/LOX-inhibiting compound is licofelone (ML-3000) (**Fig. 15)**. Licofelone inhibits COX-1 as well as FLAP and has anti-analgesic, anti-platelet and anti-inflammatory activity. The gastroinstetinal side-effects were significantly lower compared to naproxen [227]. Licofelone was investigated in several clinical studies, for example in osteoarthritis. Moreover, licofelone reduced mPGES-1-derived PGE_2 formation in the low micromolar range in celllular and cell-free assays. Interestingly, COX-2-derived product formation was unaffected by licofelone *in vitro* [228].

Thus, dual inhibition of 5-LO and mPGES-1 appears superior over dual inhibition of COX and 5-LO, since mPGES-1 converts only COX-2-derived PGH_2 to "pro-inflammatory" PGE_2, while the conversion of PGH_2 by other PGE

synthases is not altered. Concomitant inhibition of COX-2 and 5-LO or 5-LO / mPGES-1 seems to have a protective role in cancer [40]. Recently, synthetic pirinixic acid derivatives [229], benzo[g]indoles [230] and the acylphloroglucinol hyperforin [231] from plant origin were described as dual inhibitors of 5-LO and mPGES-1.

2 Aim of the study

5-LO is the key enzyme for the biosynthesis of LTA_4, which is further enzymatically converted to LTB_4 and to the cysteinyl-LTs. Leukotrienes are arachidonic-derived lipid mediators with major roles in inflammatory diseases such as asthma, allergic rhinitis, rheumatoid arthritis [60] and also in atherosclerosis and other cardiovascular diseases [176], carcinogenesis [182] and neurological disease [232]. These wide-ranging pathophysiological implications require the development of specific leukotriene biosynthesis inhibitors for the treatment of the aforementioned diseases.

Currently, the direct iron-ligand 5-LO inhibitor zileuton and $CysLT_1$ receptor antagonists such as montelukast are approved drugs for the treatment of asthma and allergic rhinitis. However, the side effects of zileuton [196], an insufficient efficacy of the $CysLT_1$ antagonists compared to inhaled corticosteroids in moderate to severe asthma and non-responders to anti-LT treatment [81] limited the success of these substance classes. The enzymatic activity of 5-LO and LT biosynthesis are regulated in a complex manner in cell-free assays and in the cell, which offers several points of attack for inhibitors. The catalytic activity of 5-LO is influenced by Ca^{2+}, ATP, glycerides, phospholipids, CLP, lipid peroxides and by phosphorylation of kinases. Moreover, in the cellular environment a compound might interfere with $cPLA_2$, FLAP, the subcellular distribution of 5-LO, its catalytic cycle or other enzymes involved in LT biosynthesis resulting in reduced LT formation. This complex regulation of LT biosynthesis must be taken into account for *in vitro* experiments aiming to evaluate the pharmacological potential of a given test compound.

In this study, different sets of compounds were designed and synthesized in cooperation with groups at the University of Erlangen, University of Salerno, University of Ankara and University of Tübingen. The structural design of the compounds was carried out by modification of natural or synthetic compounds

with known anti-inflammatory activity. For one set of compounds, a virtual screening approach using a ligand-based pharmacophore model was applied.

In order to characterize the inhibitory action of the compound on 5-LO product formation *in vitro*, cell-based and cell-free test systems and a whole blood assay were used. For selected compounds, *in vivo* studies in different animal inflammation models were carried out in cooperation with other groups. In addition, one class of compounds was further characterized in molecular docking studies to define chemical interactions of the compounds with 5-LO. Moreover, the influence of some compounds on PG formation was studied. Thus, the inhibition of COX-1, COX-2 and mPGES-1 product formation was studied in cell-based and cell-free assays. Some compounds turned out to affect COX and 5-LO or mPGES-1 and 5-LO, which may enhance their anti-inflammatory properties.

Taken together, this study presents the identification of diverse leukotriene biosynthesis inhibitors. In addition, SAR studies and mechanistical investigations were carried out to elucidate the inhibitory effect of the compounds on LT and PG formation and the anti-inflammatory actions of selected compounds were confirmed *in vivo*.

3 Material and Methods

3.1 Chemicals

Acrylamide solution 30% (37.5:1),Tris: Applichem (Darmstadt, Germany),; DMSO, SDS, Triton X-100, Tween 20: Carl Roth (Karlsruhe, Germany); IPTG, Leupeptin: Applichem (Darmstadt, Germany); Lymphocyte separation medium LSM 1077, DMEM/RPMI 1640 (cell culture medium): PAA (Coelbe, Germany); PeqGold Protein Marker IV: peqLab Biotechnology (Erlangen, Germany). HPLC solvents were from Merck (Darmstadt, Germany). All other chemicals were purchased from Sigma Aldrich (Deisenhofen, Germany), unless stated otherwise. Cell culture material was obtained from Greiner (Nürtingen, Germany)

3.2 Test compounds

Indole- and benzo[g]indole-3-carboxylates were synthesized by Dr. Eva Haberl as part of her doctoral thesis and obtained from the group of Prof. Dr. R. Troschütz (University of Erlangen, Germany). 1,4-Benzoquinone and polyphenolic compounds were synthesized and obtained from the group of Dr. Rosanna Filosa (University of Salerno, Italy). Derivatives of pyrazole-3-propanoic acid and derivatives of compounds **76** and **83** were synthesized and obtained from the group of Prof. Dr. Erden Banoglu (University of Ankara, Turkey). The test compounds described in chapter **4.4** were obtained from Ambinter Chemicals (Paris, France).

All test compounds were dissolved at 10 or 30 mM in DMSO or ethanol and kept in the dark at -20 °C and freezing-thawing cycles were kept to a minimum.

3.3 Primary antibodies

Table 1 primary antibodies used for Western blotting
source of antibody: (m) mouse, (r) rabbit

antibody (source)	**supplier**	**dilution**
5-LO (m)	provided by Prof. D. Steinhilber, (Frankfurt/Main, Germany)	1:4 to 1:8 in TBS
ERK (r)	Cell Signaling (Boston, MA, USA)	1:1,000 in 5% BSA-TBS-T
p-ERK (m) (Thr202/Tyr204)	Cell Signaling (Boston, MA, USA)	1:1,000 in 5% BSA-TBS-T
p-p38 MAPK (r) (Thr180/Tyr182)	Cell Signaling (Boston, MA, USA)	1:1,000 in 5% BSA-TBS-T

3.4 Secondary antibodies

Table 2 secondary antibodies used for Western blotting

antibody	**supplier**	**dilution**
anti-mouse-IgG anti-rabbit-IgG (alkaline phosphatase-conjugated)	Sigma-Aldrich (Deisenhofen, Germany)	1:1,000 in TBS
anti-mouse IgG (Cy3-conjugated) anti-rabbit IgG (Cy5- conjugated)	GE Healthcare/Amersham (Munich, Germany)	1:2,500 in TBS-T 1:2,500 in TBS-T

3.5 Methods

3.5.1 Isolation of PMNL and platelets from buffy coats

Human polymorphonuclear leucocytes (PMNL) and platelets were freshly isolated from leukocyte concentrates (buffy coats) obtained at Blood Center, University Hospital, Tübingen, Germany. In brief, venous blood was subjected to centrifugation at 4,000 x g for 20 minutes at 20 °C for preparation of leukocyte concentrates. Buffy coats were pooled and diluted 1:1 (v/v) with phosphate buffered saline (PBS), pH 7.4 (1.06 mM KH_2PO_4, 3 mM Na_2HPO_4, 154 mM NaCl) and then 1:4 (v/v) with dextrane solution (5% in PBS) to allow erythrocyte sedimentation for 45 minutes. After sedimentation, cells were further separated by centrifugation (1,000 x g, 10 min at RT w/o brake) on LSM 1077 Lymphocyte separation medium cushions. PMNL were collected and resuspended in PBS, pH 7.4 followed by hypotonic lysis of erythrocytes and twice washing in PBS and centrifugation steps. PMNL were finally resuspended in PBS containing 1 mg/ml glucose (PG buffer) or in PBS containing 1 mg/ml glucose and 1 mM $CaCl_2$ (PGC buffer). For isolation of platelets, platelet-rich-plasma (PRP) was obtained from the supernatants, mixed with PBS, pH 5.9 and centrifuged (2100 × g, 15 min, RT). The pelleted platelets were washed in PBS, pH 5.9 / 0.9% NaCl (1:1, v/v), centrifuged again, and finally resuspended in PBS pH 7.4. Cells were counted after addition of trypan blue solution (0.2% (w/v) trypan blue, 0.9% (w/v) NaCl) under a light microscope using a "Bürker" haemocytometer.

3.5.2 Determination of 5-, 12- and 15-LO product formation in PMNL

PMNL (5 x 10^6 to 10^7/ ml) were finally resuspended in PGC buffer and cells were pre-incubated with the test compounds or vehicle for 15 minutes at 37 °C. 5-LO product formation was started by addition of 2.5 µM Ca^{2+}-ionophore A23187 alone or with the indicated concentrations of AA in 2.5 µl of methanol. When NaCl was used for stimulation, 100 µl of 3 M NaCl solution (final concentration: 0.3 M NaCl) was added 3 minutes prior to AA.

In experiments with Ca^{2+}-depletion, 5 x 10^6 /ml PMNL were resuspended in PBS pH 7.4 containing 1 mM EDTA and pre-incubated with 30 µM BAPTA-AM for 15 minutes prior to pre-incubation with the compounds for 15 minutes at 37 °C and then stimulated with 50 µM AA. After 10 minutes at 37 °C, the reaction was stopped with 1 ml of ice-cold methanol and 500 µl of PBS, 30 µl 1 M HCl and 200 ng of prostaglandin B_1 (internal standard) were added. The samples were centrifuged (800 x g, 10 min), and the supernatant was applied to C18 solid-phase extraction columns, pre-conditioned with 1 ml of methanol and 1 ml of water. After washing with 1 ml of water and 1 ml of 25% (v/v) methanol, AA metabolites were eluted with 300 µl methanol and diluted with 120 µl water. 50-100 µl extract was analyzed by HPLC on a NovaPak® C18-column (5 x 100 mm, 4 µm particle size, Waters, Eschborn, Germany). HPLC analysis was carried out at a flow rate of 1.2 ml/min. The mobile phase consisted of MeOH / H_2O / TFA (76/24/0.007 (v/v)). 12(S)-H(P)ETE, 15(S)-H(P)ETE and 5-LO products and were detected by diode array detector or UV detector at 235 nm (5-,12-,15-H(P)ETE)) and 280 nm (PGB_1, LTB_4 and isomers). 5-LO product formation includes LTB_4 and its all-trans isomers, 5(S),12(S)-di-hydroxy-6,10-trans-8,14-cis-eicosatetraenoic acid [5(S),12(S)-DiHETE], and 5(S)-hydro(peroxy)-6-trans-8,11,14-cis-eicosatetra-enoic acid [5-H(p)ETE]. 5-LO product formation is expressed as percentage of remaining product formation of DMSO control. DMSO concentration never exceeded 0.5%. 12(S)-H(P)ETE and 15(S)-H(P)ETE were also analyzed in cellular systems to investigate inhibition of p12-LO and 15-LO. Cys-LTs (LTC_4, D_4, and E_4) and oxidation products of LTB_4 were not determined. Quantification of AA metabolites was done by peak area integration and comparing the peak areas with that of internal standard PGB_1 after correction of the different extinction coefficients of the compounds.

3.5.3 Determination of 5-LO product formation in cell homogenates

For determination of 5-LO product formation in homogenates, 1 mM EDTA was added to cells resuspended in PBS buffer, pH 7.4. Samples were cooled on ice for 5 minutes and homogenized by sonification (3 × 10 sec) at 4 °C with a

Branson cell disruptor model B15. After addition of 1 mM ATP, 5 mM GSH or 1 mM DTT were added as indicated and aliquots of 1 ml were pre-incubated 10 minutes at 4 °C with vehicle (max. 0.3% DMSO = 100% control) or compound, pre-warmed for 30 seconds at 37 °C and then 2 mM $CaCl_2$ and the indicated concentrations of AA were added to start the reaction. After 10 minutes at 37 °C, the reaction was stopped with 1 ml of ice-cold methanol and further steps and analysis were performed as described for intact cells.

3.5.4 Expression and purification of recombinant human 5-lipoxygenase

Human recombinant 5-lipoxygenase (5-LO) was obtained from E.coli (BL 21) transfected with pT3-5-LO plasmid. The bacteria culture was incubated over night under shaking at 37 °C in LB-medium supplemented with ampicillin (100 µg/ml). The bacteria were reseeded in LB-medium containing ampicillin (100 µg/ml), $FeSO_4$ (10 µM) and $MgSO_4$ (1 mM). Following incubation for 4 h at 30 °C, the expression of 5-LO was induced by IPTG (190 µg/ml). The expression was continued over night under the same conditions. For harvesting, bacteria were centrifuged (7700 x g, 15 min, 4 °C) and the resulting pellet was resuspended in lysis buffer (1 mg/ml lysozyme, 50 mM TEA pH 8, 5 mM EDTA, 60 µg/ml STI, 2 mM DTT, 1 mM PMSF) and stored on ice for 30 minutes. To complete disruption and lysis of the cells the samples were sonicated (3 x 15 sec) with a Branson cell disruptor model B15 followed by centrifugation (40,000 x g, 20 minutes, 4 °C). The resulting supernatant is referred to as S40 and was immediately used for inhibition assays.

For some experiments, 5-LO was further purified by affinity chromatography using ATP-agarose columns (Sigma A2757, bed volume 2 ml). The column was equilibrated with PBS buffer, pH 7.4 containing 1 mM EDTA. After application of the 40,000 x g supernatant, the column was first washed with 7 ml of equilibration buffer followed by 10 ml of phosphate buffer containing 0.5 M NaCl (50 mM phosphate, pH 7.4, 1 mM EDTA, 0.5 M NaCl) and washed with 10 ml of phosphate buffer (50 mM phosphate, pH 7.4, 1 mM EDTA). Finally, bound 5-LO was eluted with phosphate buffer containing 20 mM ATP (50 mM

phosphate, pH 7.4, 1 mM EDTA, 20 mM ATP). Purified 5-LO was diluted with PBS buffer, pH 7.4 containing EDTA (1 mM) and immediately used for inhibition assays.

3.5.5 Determination of 5-LO product formation in cell-free assay (recombinant 5-LO)

Aliquots (1 ml) of S40 or purified 5-LO as indicated were supplemented with 1 mM ATP and pre-incubated for 10 minutes at 4 °C with vehicle (max. 0.3% DMSO) or compound and pre-warmed for 30 seconds at 37 °C. Then, 2 mM $CaCl_2$ and AA at the indicated concentrations were added. The reaction was stopped after 10 minutes at 37 °C by addition of 1 ml ice-cold methanol. Further steps and HPLC analysis were carried out as described for intact cells.

3.5.6 DPPH assay

Antioxidant activity of test compounds was assessed by the method of Blois [233], with slight modifications. Briefly, 100 µl of 1, 2.5, 5, 10, 25, 50 or 100 µM test compound in ethanol (corresponding to 0.1, 0.25, 0.5, 1, 2.5, 5 or 10 nmoles) were added to 100 µl of a solution of the stable free radical diphenylpicrylhydrazyl (DPPH) in ethanol (100 µM, corresponding to 10 nmoles), buffered with acetate to pH 5.5, in a 96-well plate. The absorbance was read at 520 nm after 30 min incubation under gentle shaking in the dark. Ascorbic acid and L-cysteine were used as reference compounds and reduced the DPPH radical with a 1:2 (ascorbic acid : DPPH) or 1:1 (L-cysteine:DPPH) apparent stoichiometry. All analyses were performed at least in duplicates.

3.5.7 Determination of 5-LO product formation in whole blood

Aliquots of freshly withdrawn blood (1.5 ml) were pre-incubated with the compound or vehicle (0.1% DMSO) for 10 minutes at 37 °C and formation of LO products was started by addition of 30 µM Ca^{2+}-ionophore A23187. Samples were further incubated for 10 minutes at 37 °C and the reaction was stopped on ice. The samples were placed on ice, centrifuged (600 × g, 10 min, 4 °C) and aliquots of the resulting plasma (500 µl) were then mixed with 2 ml of methanol,

and 200 ng prostaglandin B1 were added as internal standard. The samples were placed at -20 °C for 2 h and centrifuged again (600 × g, 15 min, 4 °C). The supernatants were collected, diluted with 2.5 ml PBS buffer, pH 7.4 and 75 µl 1N HCl. Formed LO metabolites were extracted by solid phase extraction on C18-colums using two washing steps and analyzed by HPLC as described above for intact cells.

3.5.8 Determination of COX-1 activity in intact platelets

As COX-1 is the major enzyme in human platelets converting AA into PGH_2 which is metabolized to 12-HHT in an nonenzymatically process [234], it was used as parameter to determine COX-1 activity. Freshly isolated platelets (10^8/ml in PGC buffer) were pre-incubated with the compounds for 4 minutes at 4 °C followed by 1 minute at 37 °C. After addition of 5 µM AA and further incubation for 5 minutes at 37 °C, the reaction was stopped by adding 1 ml ice-cold methanol. 12-HHT was extracted by solid phase extraction and analyzed by HPLC (detection at 235 nm) as described above.

3.5.9 Determination of COX-2 product formation in intact A 549 cells

2×10^6 cells were plated in 175 cm^2 flasks and incubated for 16 h at 37 °C and 5 % CO_2 atmosphere. Thereafter, medium was replaced by fresh DMEM/High glucose (4.5 g/l) medium containing 2% FCS and cells were stimulated with interleukin-1β (1 ng/ml) for 72 h. After trypsination, the cells were washed twice with PBS buffer, pH 7.4 and counted after addition of trypan blue solution 1:1 (v/v) (0.2% trypan blue, 0.9% NaCl) under a light microscope using a Bürker haemocytometer.

For determination of 6-keto-$PGF_{1\alpha}$, 10^6 cells/ml in PGC buffer were pre-incubated with the compounds for 15 minutes at 37 °C. 6-keto-$PGF_{1\alpha}$ formation was initiated by addition of 3 µM AA. After 15 minutes at 37 °C, the reaction was stopped by cooling on ice. Cells were centrifuged (300 × g, 5 min, 4 °C) and the amount of released 6-keto $PGF_{1\alpha}$ in the supernatant was quantified using

a 6-keto-$PGF_{1\alpha}$ High Sensitivity EIA Kit (Assay Designs, Ann Arbor, MI) according to the manufacturer's protocol.

3.5.10 Determination of mPGES-1 product formation

For this assay, all steps were performed on ice due to the instability of the reagents. Microsomes of A549 cells stimulated with IL-1β expressing mPGES-1 were diluted in homogenization buffer (phosphate buffer (0.2 M, pH 7.4) containing 0.25 M sucrose, 1 mM PMSF, 60 µg/ml STI, 10 µg/ml leupeptin and 2.5 mM GSH). 50 µl of the diluted microsomes were plated into a 96-well-plate and pre-incubated for 15 minutes with test compounds or DMSO (max. 1%) and the control inhibitor MK-886. The samples were incubated with 20 µM PGH_2 for 60 sec. Then, the reaction was stopped by adding 100 µl stop solution (40 mM $FeCl_2$, 80 mM citric acid) containing the internal standard 11β-PGE_2 (10 µM). The samples were pre-cleared by solid phase extraction using RP-18 columns conditioned with acetonitrile and water. The columns were washed with 2 x 500 µl of water and PGE_2 was eluted with 200 µl of acetonitrile. Afterwards the eluate was diluted with 400 µl of water. Subsequently the PGE_2 formation was quantified via HPLC at 195 nm with a mobile phase consisting of acetonitrile / H_2O / TFA (31.5 / 68.5 / 0.007 (v/v)).

3.5.11 Subcellular localization of 5-LO by mild detergent lysis

For studies of subcellular 5-LO distribution, freshly isolated human PMNL (3×10^7 /ml) from female donors were resuspended in 1 ml of cold PGC buffer. Cells were pre-incubated for 15 minutes at 37 °C with compound or DMSO (max. 0.3%). Then, 2.5 µM Ca^{2+}-ionophore A23187 was added and samples were incubated for further 5 minutes at 37 °C. To stop the reaction, samples were chilled on ice. After 5 minutes on ice, samples were centrifuged (200 x g, 5 minutes, 4 °C). The supernatant was discarded and pellets were resuspended in 300 µL ice-cold NP-40 lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 0.1% NP-40, 1 mM PMSF, 60 µg/ml STI, 10 µg/ml leupeptin). For lysis, samples were vortexed (3 x 5 seconds), kept on ice for 10 minutes, and centrifuged again (1,000 x g, 10 minutes, 4 °C).

Resulting supernatants (non-nuclear fractions) were transferred to a new tube. The pellets containing the nuclear fractions were resuspended in 300 µl ice-cold relaxation buffer (50 mM Tris-HCl pH 7.4, 250 mM sucrose, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF, 60 µg/ml STI, 10 µg/ml leupeptin). Both nuclear and non-nuclear fractions were centrifuged again (1,000 x g, 10 min, 4 °C) for further purification. Lysis of cells and integrity of nuclei were confirmed by light microscopy with trypan blue exclusion. Nuclear fractions in relaxation buffer were disrupted by sonication on ice (3 x 5 seconds) with a cell disruptor B15 Branson sonifier. Aliquots of nuclear and nonnuclear fractions were immediately mixed with the same volume of cold 2× SDS loading buffer (20 mM Tris-HCl pH 8, 2 mM EDTA, 5% SDS, and 10% β-mercaptoethanol) and heated for 6 minutes at 95 °C. Distribution of 5-LO in both fractions was analyzed by SDS-PAGE and Western blotting.

3.5.12 Determination of p42/44 MAPK (ERK) and p38 MAPK activation

Freshly isolated PMNL (5×10^6) from female donors were resuspended in PGC buffer to a final volume of 100 µl. After pre-warming of the samples for 3 minutes at 37 °C, followed by pre-incubation with the compounds or DMSO (max. 1%) for 15 minutes, stimulation with 0.1 µM fMLP, was performed for 3 minutes at 37 °C. The reaction was stopped by addition of 100 µl of cold 2 × SDS loading buffer (20 mM Tris-HCl pH 8, 2 mM EDTA, 5% SDS, 10% β-mercaptoethanol) and samples were heated for 6 min at 95 °C. Phosphorylation of p42/44-MAPK (Thr202/Tyr204) and p38 MAPK (Thr180/Tyr182) was analyzed by SDS-PAGE and Western blotting.

3.5.13 SDS-PAGE

20 µl of protein samples were mixed with 4 µl of bromophenol blue solution (50% (v/v) glycerol, 0.1% (w/v) bromophenol blue) and separated by SDS-PAGE using a MiniProtean System (Bio-Rad, Hercules, CA, USA). Polyacrylamid concentration of the gels was 10% for subcellular distribution of 5-LO and 12% for investigation of phosphorylated kinases. Molecular weight of the investigated proteins was estimated by comparison with prestained broad

range protein marker peqGOLD IV. For good separation, 90 Volt for the stacking gel and 120-140 Volt for the separation gel was used.

3.5.14 Western Blotting

After separation of the proteins by SDS-PAGE, proteins were blotted (tank blotting method) from polyacrylamide gels to nitrocellulose membranes (90 V for 90 min, in transfer buffer (48 mM Tris, 40 mM glycine, 20% (v/v) methanol, 0.1 mM SDS). For investigation of phosphorylated proteins, transfer buffer without SDS was used. After electroblotting to a nitrocellulose membrane (GE Healthcare, Munich, Germany), correct loading of the gel and transfer of proteins was confirmed by staining the membrane with Ponceau S solution (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid). Then, membranes were blocked with 5% BSA in blocking buffer (50 mM Tris/HCl, pH 7.4 and 100 mM NaCl (TBS plus 0.1% Tween 20 (= TBS-T)) for 1 h at RT. Membranes were washed again with TBS-T and incubated with the respective diluted primary antibodies overnight at 4 °C (for antibody dilutions **see Table 1).**

Membranes were washed 3 times with TBS-T and incubated with respective secondary antibodies diluted in TBS-T (**Table 2**) Alkaline phosphatase-conjugated IgG were applied for 2 h at RT or CyDye-conjugated secondary antibodies for 1 h at RT. Incubation with CyDye-conjugated antibodies and washing steps were performed in the dark to avoid bleaching of the fluorophoric group. After washing, proteins were visualized by NBT/BCIP detection for alkaline phosphatase-conjugated antibody. Alternatively, an ETTANDIGE Imaging system (GE Healthcare), was used for CyDye-conjugated antibodies using Cy3 (excitation filter: 540 nm; emission filter: 595 nm) and Cy5 (excitation filter: 635 nm; emission filter: 680 nm) channels. Densitometric analysis of the bands was performed by ImageQuant^{TM}TL Software (GE Healthcare) as indicated.

3.5.15 Isolation of monocytes and determination of $cPLA_2$ inhibition

Fresh blood was collected in heparinized tubes (16 I.E. heparin/ml blood) by venipuncture from fasted (12 h) adult male and female healthy volunteers, with consent (Blood Center, Jena, Germany). The subjects had no apparent inflammatory conditions and had not taken oral contraceptives (or other sex hormones) or anti-inflammatory drugs for at least ten days prior to blood collection. The blood was subjected to centrifugation (4,000 x g, 20 min, 20 °C) and peripheral blood mononuclear cells were promptly isolated by dextran sedimentation and centrifugation on LSM 1077 Lymphocyte separation medium cushions. Cells were collected, washed three times with cold PBS buffer, pH 7.4 and then monocytes were separated by adherence for 1 h at 37 °C to culture flasks (Greiner, Nuertingen, Germany; cell density: 20 x 10^6 cells/ml in RPMI 1640 medium containing 2 mM L-glutamine and 50 µg/ml penicillin / streptomycin), which gave a purity of > 85%, defined by forward- and side-light scatter properties and detection of the CD14 surface molecule by flow cytometry (BD FACS Calibur). Monocytes were finally resuspended in ice-cold PG buffer or PGC buffer. Monocytes (2×10^6 /ml RPMI) were incubated with 5 nM [^{3}H]-arachidonic acid for 2 h at 37 °C and 6% CO_2. Cells were washed twice with PBS buffer, pH 7.4 containing 1 mg/ml glucose and 2 mg/ml BSA, resuspended in PG buffer (5×10^6 /ml), supplemented with 1 mM $CaCl_2$, pre-incubated with the test compounds for 15 minutes at 37 °C and stimulated with 1 µM Ca^{2+}-ionophore A23187 for 5 minutes at 37 °C. The samples were stopped on ice for 10 minutes and centrifuged at 1200 × g, 10 min. Aliquots of the supernatants were mixed with 2 ml Ultima GoldTM XR and measured on a scintillation counter (Micro Beta Trilux, Perkin Elmer, Waltham, MA, USA) to detect released [^{3}H]-AA.

3.5.16 Expression of recombinant $cPLA_2\alpha$ and determination of $cPLA_2$ inhibition

The $cPLA_{2\alpha}$ coding sequence was cloned from pVL1393 plasmid (kindly provided by Dr. Wonhwa Cho, University of Illinois at Chicago) into

pFastBacTM HT A containing a 6 x his-tag coding sequence. The recombinant plasmid was transformed into DH10BacTM E. coli. Sf9 cells were transfected with recombinant bacmid DNA using Cellfectin® Reagent and the generated baculovirus was amplified. Overexpression of His-tagged $cPLA_2$ in baculovirus-infected Sf9 cells and isolation using Ni-NTA agarose beads was performed as described [23].

Multilamelar vesicles (MLVs) were prepared by drying 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphocholine (PAPC) and 1-palmitoyl-2-oleoyl-sn-glycerol (POG) in a ratio of 2:1 (in chloroform) under nitrogen in glass vials. After addition of 20 mM Tris buffer, pH 7.4 containing 134 mM NaCl and 1 mg/ml fatty acid free BSA, the MLV suspension was disrupted by several freeze-thaw cycles (liquid nitrogen) and then extruded 11 times with a mini-extruder (Avanti Polar Lipids, Inc) through a polycarbonate membrane (100 nm pore diameter) at RT (above transition temperature of the lipids) to produce large unilamelar vesicles (LUV). Final total concentration of lipids was 250 µM in 200 µl. Test compounds and 1 mM $CaCl_2$ were added to the vesicles, and the reaction was started by addition of 500 ng his-tagged $cPLA_2$ (in 10 µl buffer). After 1 h at 37 °C, 1.6 ml methanol was added, and AA was extracted by RP-18 solid phase extraction. Following derivatisation with p-anisidinium chloride, the , the resulting derivate was analyzed by RP-HPLC at 249 nm as described [235].

3.5.17 Carrageenan-induced pleurisy in rats

Male Wistar Han rats (220-230 g, Harlan, Milan, Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care complied with Italian regulations on protection of animals used for experimental and other scientific purpose (Ministerial Decree 116192) as well as with the European Economic Community regulations (Official Journal of E.C. L 358/1 12/18/1986).

Compounds were given i.p. 30 minutes before carrageenan, whereas vehicle-treated group of rats received DMSO (4%, i.p.) 30 minutes before carrageenan.

Rats were anaesthetized 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 (isolated from *Gigartina aciculaire* and *Gigartina pistillata*, Sigma-Aldrich, Milan, Italy) 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 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 under a light microscope using a Bürker haemocytometer after vital trypan blue staining. The amount of LTB_4 in the supernatant of centrifuged exudate (800 x *g,* 10 min) was assayed by EIA immunoassay (Cayman Chemical, Ann Arbor, MI) according to manufacturer's protocol. The results are expressed as nanograms per rat and represent the mean ± SEM of rats per experimental group as indicated.

4 Results

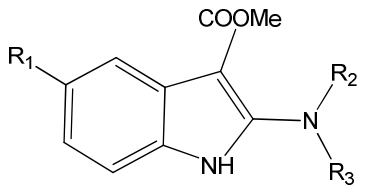
4.1 2-substituted-indole-3-carboxylates as 5-LO inhibitors

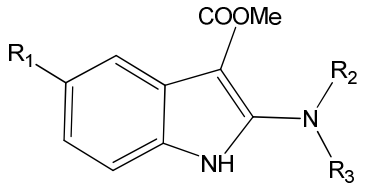
Synthesis and biological evaluation of a series of 2-amino-5-hydroxy-indole-3-carboxylate derivatives as inhibitors of 5-LO was recently reported [236]. No 5-LO inhibition was found for compounds with a primary amino group or dimethylamine moiety in position 2 at the 5-hydroxyindole nor for compounds including the nitrogen atom of the amino group into heterocycles as pyrrolidine, piperidine, methylpiperazine or morpholine, up to a concentration of 30 µM. Introduction of 4-arylpiperazine residues successfully generated 5-LO inhibitory properties. Especially a (4-chlorophenyl)piperazin-1-yl derivative blocked 5-LO product formation efficiently at 10 µM. The potency of the 4-arylpiperazine derivatives proved to be higher in the cell-free assay than in intact cells, suggesting low cell-permeability due to positive charging of the basic piperazine structure. Synthesis of another series of compounds containing secondary phenyl- and phenylethylamine moieties lead to even more potent compounds [236]. One of the most active compounds identified was methyl 2-(3-chlorophenylamino)-5-hydroxy-1H-indole-3-carboxylate (**1a**) (IC_{50}= 2.4 µM in A23187-stimulated PMNL and 0.3 µM in 40,000 x g homogenates of E.coli with recombinant 5-LO (referred to as S40), respectively.

4.1.1 SAR of indole-carboxylates

The hydroxy group in position 5 of the indole backbone is assumed to have anti-oxidative properties and thus explaining the inhibitory effect on 5-LO by keeping the catalytic iron in the ferrous (Fe^{2+}) state. In order to determine the influence of the 5-hydroxy moiety on the potency of the respective substance, several derivatives devoid of it were synthesized in the group of Prof. Dr. Troschütz at University of Erlangen and tested for their 5-LO inhibition (**Table 3**).

Table 3 5-LO inhibition of 2-amino-indole-3-methylcarboxylates
PMNL were stimulated with 2.5 µM A23187+ 20 µM AA or to aliquots of 40,000x g homogenates of E.coli expressing recombinant 5-LO (S40), 1 mM $CaCl_2$ and 20 µM AA were added. IC_{50} values are given as mean ± SEM; n ≥ 3
** PMNL stimulated with 2.5 µM A23187 n.i. [a]: no inhibition up to 10 µM;*

				**inhibition of 5-LO activity IC_{50} [µM ± SEM]**	
#	**R1**	**R2**	**R3**	**PMNL**	**S40**
1a	-OH	-H	3-chlorophenyl	7.3 ± 0.9 2.4 ± 0.4 *	0.3 ± 0.09
1b	-H	-H	3-chlorophenyl	6.5 ± 1.9	8.1 ± 0.5
1c	-Cl	-H	3-chlorophenyl	5.5 ± 2.1	3.5 ± 0.9
1d	-H	$-CH_3$	3-chlorophenyl	2.4 ± 0.3	n.i. [a]
2a	-H	-H	2-chlorophenyl	9.8 ± 1.9	10.2 ± 0.5
2b	-H	-H	4-chlorophenyl	7.2 ± 0.9	9.8 ± 4.0
2c	-H	-H	3-fluorophenyl	7.9 ± 0.3	13.4 ± 2.9
2d	-H	-H	2-bromophenyl	7.1 ± 0.5	7.6 ± 0.4
2e	-H	-H	3-bromophenyl	3.5 ± 1.2	8.3 ± 1.4
2f	-H	-H	2-chloro-(3-pyridinyl)	2.4 ± 0.2	1.4 ± 0.3
3a	-H	-H	-benzyl	4.0 ± 2.8	3.4 ± 1.8
3b	-H	-H	-allyl	n.i. [a]	n.i. [a]
3c	-H	-allyl	-allyl	12.5 ± 2.5	6.9 ± 1.4
4	-H	-H	1-naphtyl	3.6 ± 0.1	10.2 ± 0.3
5a	-H	-H	3,5-dichlorophenyl	7.8 ± 0.9	2.9 ± 1.1
5b	-H	-H	3,4-dichlorophenyl	4.6 ± 0.8	4.5 ± 1.3
5c	-H	-H	2,6-dichlorophenyl	7.9 ± 1.9	8.5 ± 1.0

				**inhibition of 5-LO activity IC_{50} [µM ± SEM]**	
#	**R1**	**R2**	**R3**	**PMNL**	**S40**
5d	-H	-H	3-trifluormethylphenyl	6.5 ± 0.5	9.7 ± 2.5

In direct comparison to parental compound **1a**, the derivative lacking the 5-hydroxy moiety (**1b**) was equipotent in PMNL, but 30-fold less potent in the cell-free assay. Replacement of the hydroxy group by chlorine (**1c**) did not significantly impair the efficacy in intact cells, but lowered the potency12-fold in the cell-free assay. When a methyl group was present at the amino moiety (**1d**), inhibition in the cell-free assay was diminished, whereas the potency in PMNL was enhanced almost 3-fold.

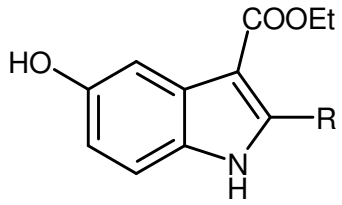
Variation of the substituents at the aromatic ring by chlorine in ortho- (**2a**) or para-position (**2b**) as well as flourine in meta- (**2c**) or bromine in ortho-position (**2d**) caused no substantial improvement versus **1b**, except meta-positioning of bromine (**2e**) (IC_{50}= 3.5 ± 1.2 µM). Replacement of 2-chlorophenyl (**2a**) by 2-chloro-3-pyridinyl (**2f**), increased the potency 4-fold in PMNL and 7-fold in the cell-free assay. The presence of an unsubstituted benzylamino moiety (**3a**) slightly enhanced the potency (IC_{50}= 4.0 µM in PMNL). Structural variation of the N-aryl moiety with one N-allyl chain (**3b**) led to total loss of activity in both systems whereas the N-diallyl-compound (**3c**), resembling an aromatic phenyl ring, was active [236]. Enlargement of the aromatic system to naphtyl (**4**) was not detrimental for 5-LO inhibition in intact cells but impaired potency in the cell-free assay versus **1a**.

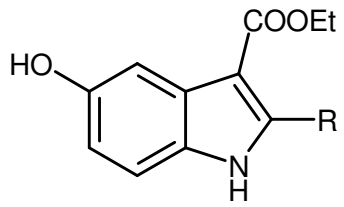
Modifications such as exchange of the nitrogen bridge of **1b** by an oxygen atom or inclusion of the nitrogen into a bicyclic phenyl-piperidinyl system (not shown), retained the inhibitory potency with IC_{50} values between 7.0 and 9.5 µM, but did not improve it. Insertion of two chlorine atoms (**5a-c**) or a meta-

positioned trifluoromethyl group (**5d**) at the phenyl moiety was tolerated leading to IC_{50} values in the range of the other phenyl derivatives from this series, whereof simultaneous chlorine substitution in meta- and para-position (**5b**) was beneficial.

Despite the lack of the hydroxy group in position 5, derivatives of 2-amino-indole-3-carboxylates (**Table 3**) inhibit 5-LO. This suggests that the inhibition of 5-LO cannot only be explained by a redox-type-mechanism of the compounds. But since the hydroxy group was shown to be beneficial, a 5-hydroxy-indole-3-ethylcarboxylate backbone was chosen for additional modifications. Moreover, substitution of the nitrogen in 2-position of the indole by carbon yielding compound **6a**, led to slightly improved potency in PMNL, but with concomitant loss of potency in the cell-free assay compared to the methyl-carboxylate analogue **1a** (**Table 4**).

Table 4 5-LO inhibition of 5-hydroxy- indole-3-ethylcarboxylates
PMNL were stimulated with 2.5 µM A23187 +20 µM AA. or to aliquots of 40,000x g homogenates of E.coli expressing recombinant 5-LO (S40), 1 mM $CaCl_2$ and 20 µM AA were added. IC_{50} values are given as mean ± SEM; n ≥ 3

		**inhibition of 5-LO activity IC_{50} [µM ± SEM]**	
#	**R**	**PMNL**	**S40**
6a	3-chlorobenzyl	1.7 ± 0.8	2.0 ± 0.5
6b	2-chlorobenzyl	1.7 ± 0.4	1.6 ± 1.6
6c	4-chlorobenzyl	0.7 ± 0.4	1.2 ± 1.1
6d	3-chlorophenyl	3.3 ± 0.6	4.8 ± 0.9
6e	4-chlorophenyl	2.0 ± 0.05	2.1 ± 0.6
6f	1-(3-chlorophenyl)ethyl	2.8 ± 0.5	1.7 ± 0.6

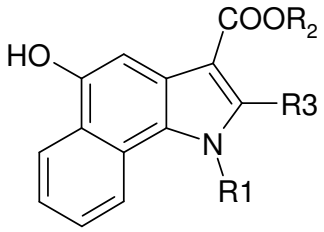
		**inhibition of 5-LO activity IC_{50} [µM ± SEM]**	
#	**R**	**PMNL**	**S40**
6g	1-(4-chlorophenyl)ethyl	3.9 ± 1.8	0.7 ± 0.3
6h	1-phenylethyl	5.5 ± 1.4	7.3 ± 1.6
6i	2-chloro-6-fluorophenylamino	7.9 ± 2.5	1.6 ± 0.1

Repositioning of chlorine to para position (**6c**) at the aromatic ring increased the potency about 2-fold to 0.7 µM ± 0.4 in PMNL and 1.2 ± 1.1 µM in the cell-free system, whereas ortho-positioning (**6b**) was equipotent with **6a**. When a bulky benzyl residue was attached to the nitrogen of **6c** (not shown), a 2-fold decrease of potency in PMNL (IC_{50}= 1.6 µM), but a 12-fold increase of potency in the cell-free assay (IC_{50}= 0.1 µM) was observed. After altering the connection between the indole and the aromatic ring to chlorophenyl (**6d, 6e**), 5-LO inhibition by the 3-chlorophenyl derivative **6d** was lowered 2-fold in the cell-based and 2.5-fold in the cell-free system. For **6e**, an almost 3-fold decrease of potency was observed in intact cells and about 2-fold in the cell-free assay compared to the respective benzyl derivatives. Elongation of the bridge (**6f-h**) to 2 carbon atoms caused a slight loss of potency in PMNL compared to the corresponding benzyl analogues, still with inhibitory potency in the range of phenyl analogues. Only for **6g** an IC_{50} of 0.7 µM was observed in the cell-free system. Replacing the nitrogen at the indole part of **6h** by oxygen (not shown) yielding a benzofuran core did not improve the potency with IC_{50} values of 3.8 ± 1.8 µM and 9.7 ± 2.9 µM in PMNL and S40, respectively. The 2-chloro-6-fluorophenylamino residue at **6i** showed no substantial improvement in PMNL compared to the 2,6-dichlorophenylamino derivative **5c**. The higher potency of **6i** in the cell-free assay is likely attributed to the 5-hydroxy group.

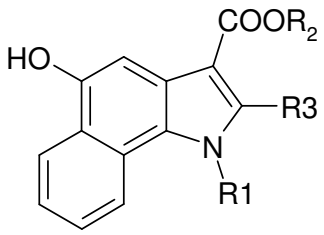
The next structural variation of the compounds involved the anellation of a benzene moiety at the indole core yielding 5-hydroxy-benzo[g]indole-3-carboxylates as new core structure (**Table 5**).

This modification enlarged the lipophilic core structure and the overall lipophilicity of the compounds. Annelation of a benzene ring to compound **1a** generated the ethylcarboxylate analogue **7** with 10-fold higher potency in PMNL compared to **1a**. Replacement of the nitrogen bridge in **7** by a methylene function leading to compound **8a** further enhanced the potency about 3-fold in both test systems. Due to the finding that the amino function in position 2 is not beneficial, synthesis of derivatives devoid of it was continued.

Table 5 5-LO inhibition of benzo[g]indole-3-carboxylates
PMNL were stimulated with 2.5 µM A23187+20 µM AA or to aliquots of 40,000 x g homogenates of E.coli expressing recombinant 5-LO (S40), 1 mM $CaCl_2$ and 20 µM AA were added. IC_{50} values are given as mean ± SEM; n ≥ 3;
Bn=Benzyl ; Et=Ethyl



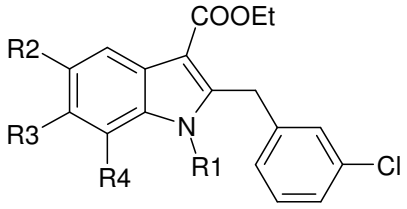
				inhibition of 5-LO activity IC_{50} [µM ± SEM]	
#	**R1**	**R2**	**R3**	**PMNL**	**S40**
7	-H	-Et	(3-chlorophenyl)amino	0.71 ± 0.25	0.24 ± 0.06
8a	-H	-Et	3-chlorobenzyl	0.23 ± 0.07	0.086 ± 0.02
8b	-H	-Et	2-chlorobenzyl	1.2 ± 0.07	0.097 ± 0.09
8c	-H	-Et	4-chlorobenzyl	1.2 ± 0.19	0.084 ± 0.05
8d	-H	-Et	3-fluorobenzyl	0.34 ± 0.03	0.14 ± 0.02
8e	-H	-Et	4-fluorobenzyl	0.50 ± 0.01	0.096 ± 0.02
8f	-H	-Et	3-bromobenzyl	0.45 ±0.14	0.15 ± 0.02

				**inhibition of 5-LO activity IC_{50} [µM ± SEM]**	
#	**R1**	**R2**	**R3**	**PMNL**	**S40**
8g	-H	-Et	4-bromobenzyl	0.60 ± 0.24	0.095 ± 0.01
8h	-H	-Et	3-methoxybenzyl	0.52 ± 0.03	0.13 ± 0.15
8i	-H	-Et	4-methoxybenzyl	0.65 ± 0.3	0.15 ± 0.02
8j	-H	-Et	4-trifluoromethylbenzyl	1.7 ± 0.3	0.25 ± 0.18
8k	-H	-Et	3-chlorophenyl	0.52 ± 0.10	0.045 ± 0.01
8l	-H	-Et	4-chlorophenyl	0.32 ± 0.11	0.067 ± 0.02
8m	-H	-Et	3-chlorophenylethyl	0.49 ± 0.15	0.031 ± 0.01
8n	-H	-Et	4-chlorophenylethyl	2.8 ± 0.6	0.049 ± 0.04
8o	-H	-Et	phenylpropyl	0.44 ± 0.11	0.13 ± 0.02
8p	-Bn	-Et	4-chlorobenzyl	1.8 ± 0.05	0.65 ± 0.09
8q	-H	-Bn	3-chlorobenzyl	0.35 ± 0.09	0.13 ± 0.09
8r	-H	-Bn	4-chlorobenzyl	0.48 ± 0.13	0.17 ± 0.02
8s	-H	-Et	2-chloro-6-fluorobenzyl	0.99 ± 0.3	0.18 ± 0.03
9	-H	-Et	(2-chloropyridin-3-yl)-methyl	0.98 ± 0.2	0.17 ± 0.05
10	-H	-Et	2-(tert-butoxycarbonylamino)ethyl	0.65 ± 0.6	0.28 ± 0.08

Different positioning of chlorine (**8a-c**), fluorine (**8d,e**), bromine (**8f,g**) or methoxy groups (**8h,i**) at the aromatic system were tolerated yieding IC_{50} values between 0.23 µM and 1.7 µM in intact cells and still very potent inhibition of 5-LO in the cell-free assay with IC_{50} values between 0.086 µM (**8a**) and 0.25 µM

for **8j**. The meta-substituted chlorobenzyl derivative **8a** was superior to flourine (**8d**) and bromine analogues (**8f**) followed by halogens in para-position (**8c, 8e, 8g**) whereof in particular chlorine substitution was even more detrimental. Introduction of methoxy groups in meta (**8h**) or para position (**8i**) as well as trifluoromethyl groups (**8j**) instead of halogens were tolerated but appeared not to be improving. Variations of the distance by shortening (**8k,l**) or elongation (**8m, n, o**) between indole core and phenyl moiety slightly improved the potency in the cell-free assay compared to **8a** (IC_{50}= 0.086 ± 0.02 µM). However, in intact cells, the potency was almost 10-fold lower when an ethylene bridge was inserted (**8n**). For the meta-substituted derivatives (**8k, 8m**) only a slight influence of chain length on inhibitory potency was observed in PMNL. Further elongation of the bridge leading to phenylpropyl-substituted **8o** did not significantly decrease potency. Introduction of a benzyl residue at N-1 of the indole at **8c** influenced potency moderately (**8p**). Synthesis of corresponding benzyl esters to **8a** and **8c** showed that even substitution with voluminous residues at position 3 of the indole (**8q, 8r**) is not critical. More than one substituent at the aromatic residue such as chlorine and fluorine (**8s**) or 2-chloro-pyridine (**9**) instead of benzene retained potency in the cell-free-assay, but slightly reduced it in PMNL to an IC_{50} of approximately 1 µM. Interestingly, bulky aliphatic substituents such as 2-(tert-butoxycarbonylamino)ethyl (**10**) were also tolerated and yielded a potency in the range of the halogenated benzyl derivatives. Based on these findings, 3-chlorobenzyl-substituted derivatives with benzo[g]indole scaffold were chosen for further modifications (**Table 6**).

Table 6 5-LO inhibition of 3-chlorobenzyl-benzo[g]indole-3-carboxylates
PMNL were stimulated with 2.5 µM A23187 + 20 µM AA. or to aliquots of 40,000x g homogenates of E.coli expressing recombinant 5-LO (S40), 1 mM $CaCl_2$ and 20 µM AA were added. IC_{50} values are given as mean ± SEM; n ≥ 3
n.i. [a]: no inhibition up to 10 µM; n.i. [b]: no inhibition up to 30 µM



#	R1	R2	R3	R4	inhibition of 5-LO activity IC_{50} [µM ± SEM] PMNL	S40
11a	allyl	-OH	benzo		n.i.[b]	> 30
11b	4-methoxy-benzyl	-OH	benzo		n.i.[a]	0.8 ± 0.5
11c	4-methoxy-benzyl	(4-trifluormethyl)-benzyloxy-	benzo		n.i.[b]	n.i.[b]
11d	-CH_3	-OCH_3	benzo		n.i.[b]	> 30
11e	-H	4-chlorophenyl	benzo		n.i.[b]	n.i.[a]
11f	-H	4-cyanophenyl	benzo		n.i.[b]	n.i.[a]
11g	-H	phenyl-	benzo		> 30	> 30
11h	-H	benzoyloxy	benzo		n.i.[a]	3.4 ± 2.0
12	-H	-OH	2,3-dimethoxybenzo		7.0 ± 0.8	> 10
13	-H	-OH	[2,3] pyrido		4.3 ± 0.9	0.58 ± 0.15
14	-H	-OH	phenyl	-H	5.7 ± 0.1	1.2 ± 0.20
15	-H	-OH	4-biphenyl	-H	2.8 ± 0.7	0.33 ± 0.12

Insertion of an allyl (**11a**) or bulky aromatic 4-methoxybenzyl (**11b**) moiety at the nitrogen of the indole as well as substitution with two bulky substituents (**11c**) at position 1 and 5 totally abolished 5-LO inhibition in cellular assay. Interestingly, compound **11b**, which was inactive in PMNL, inhibited 5-LO directly with an IC_{50} value below 1 µM. Smaller substitutents such as methyl

and methoxy groups (**11d**) showed weak inhibiton in the cell-free-assay. Replacement of the hydroxy group in position 5 with lipophilic residues such as 4-chlorophenyl (**11e**), 4-cyanophenyl (**11f**) phenyl (**11g**), or esterification with benzoic acid (**11h**) could not generate potent 5-LO inhibitors. Anellation of dimethoxybenzene (**12**) or heterocyclic pyridine (**13**) instead of benzene or introduction of a phenylic (**14**) and biphenylic (**15**) residue in C-7 position were tolerated, but proved to be detrimental for 5-LO inhibition compared to lead compound **8a**. Except for **12**, the potency of the compounds was better in cell-free-assay suggesting a lower uptake into intact cells due to bulky substituents.

4.1.2 Pharmacological characterization of selected compounds

Inhibition of 5-LO product formation might be caused by various mechanisms. Beside direct inhibition of the enzyme, competitive inhibition or influence on the activation of 5-LO are reasonable [237]. In order to assess the mechanisms of action, **8a** was chosen for further experiments.

4.1.2.1 Reversibility of 5-LO inhibition

In order to find out whether the binding of **8a** to 5-LO is reversible, a wash-out experiment was conducted. After pre-incubation of semi-purified human recombinant 5-LO with 0.5 µM of **8a**, followed by 10-fold dilution with assay buffer to 0.05 µM and addition of $CaCl_2$ and 20 µM AA, enzyme activity was recovered to 50% of vehicle control (**Fig. 16**).

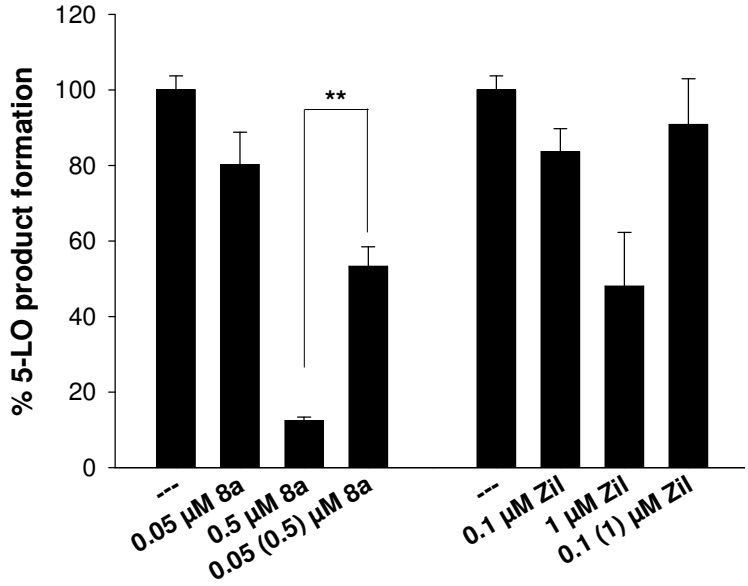


Fig. 16 Reversibility of 5-LO inhibition by 8a vs. zileuton
Aliquots of purified recombinant 5-LO were incubated with 0.05 or 0.5 µM 8a, 1 or 0.1 µM zileuton (Zil), or vehicle (DMSO) for 10 min at RT. Then, one aliquot of the sample containing 0.5 µM 8a or 1 µM Zil was diluted with assay buffer 10-fold, whereas the other aliquot was not altered. Samples were pre-warmed for 30 sec at 37 °C, and 2 mM $CaCl_2$ and 20 µM AA were added to start the 5-LO reaction. After 10 min, 5-LO products were analysed. Data shown as mean ± SEM; n = 4.

*** p< 0.01 vs. undiluted sample, one-way ANOVA with post-test*

As control inhibitor, zileuton was used at concentrations of 0.1 µM and 1 µM that inhibited 5-LO in a direct and reversible manner, as expected [194]. It has to be noted that enzyme activity was not completely reversed after dilution of **8a**. Nevertheless, **8a** can be considered as reversible inhibitor of 5-LO.

4.1.2.2 Influence of substrate concentration on 5-LO inhibition

Next, the influence of different substrate concentrations was investigated**.** After stimulation of PMNL with 2.5 µM A23187, endogenous AA is liberated from the nuclear membrane by $cPLA_2$ [20] leading to formation of LTs and 5-H(P)ETE [59]. When 20 or 50 µM AA were added together with A23187, circumventing liberation of endogenous AA by $cPLA_2$, no significant change in potency was observed (**Fig. 17A**)

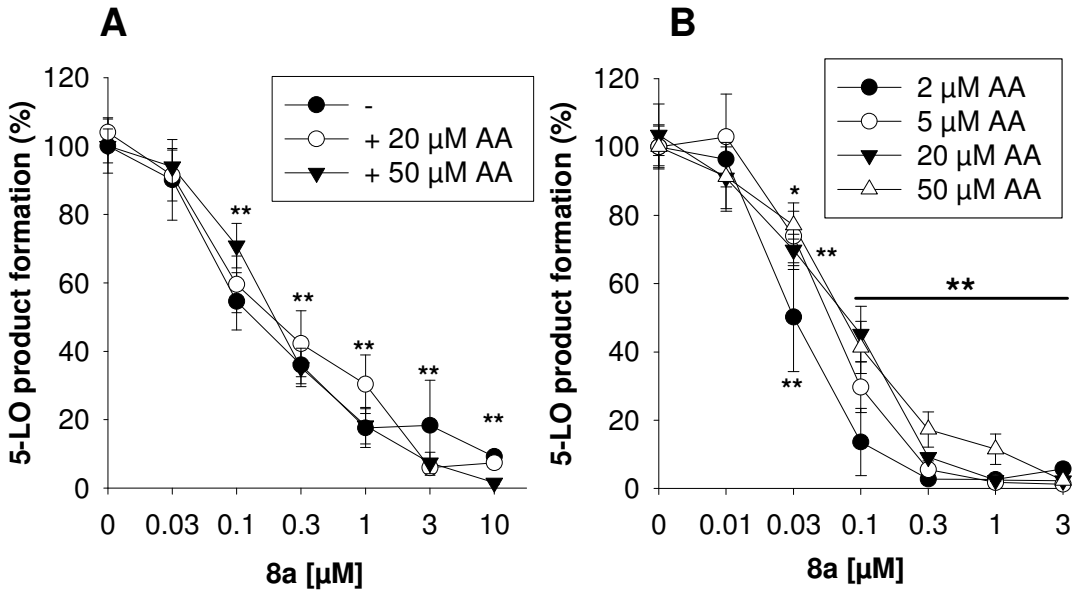


Fig. 17 Effect of different AA concentrations on 5-LO inhibition of 8a
PMNL were pre-incubated with 8a for 15 min at 37 °C. Then, 2.5 µM A23187 was added without (-) or together with 20 or 50 µM AA (A). Alternatively, aliquots of E.coli homogenates of recombinant 5-LO (S40), were diluted in 1 ml PBS, pH 7.4 and 1 mM EDTA, and pre-incubated with the test compounds for 10 min at 4 °C. Samples were pre-warmed for 30 sec at 37 °C, and 2 mM $CaCl_2$ and AA were added as indicated to start the 5-LO reaction. (B) After 10 min at 37 °C, 5-LO products were analysed by HPLC. Data shown as mean ± SEM; n =2-12;

**p<0.05, **p < 0.01 vs. control, one-way ANOVA with post-test*

Taken together, the potency of **8a** was not influenced significantly when different concentrations of AA were present in the assay suggesting a non-competitive 5-LO inhibition. Also inhibition at the stage of substrate supply ($cPLA_2$) could be excluded by this approach. Interaction of **8a** with FLAP seems also unlikely, since it has been shown that FLAP inhibitors fail to completely suppress 5-LO at high substrate concentrations. Moreover, FLAP inhibitors are not active in cell-free systems [238].

4.1.2.3 Influence on cellular distribution of 5-LO

Several FLAP inhibitors such as MK-886 contain an indole-based structure and suppress LT formation by inhibiting substrate supply mediated by FLAP. This class of leukotriene formation inhibitors was shown to inhibit translocation of 5-LO from the cytosolic compartment to the nuclear membrane and thus activation

of 5-LO [165]. Since **8a** shows structural but not mechanistical properties of FLAP inhibitors, it was not surprising that no influence on Ca^{2+}-induced 5-LO translocation was observed [239].

4.1.2.4 5-LO inhibition of 8a in whole blood assay

Physiological factors such as albumin-binding or regulatory components in blood plasma can influence the bioavailabilty of a drug *in vivo*. These effects are not considered in the assay systems used so far in this study. Hence, 5-LO inhibition by two selected 5-hydroxy-benzo[g]indole-3-carboxylates in human whole blood stimulated with 30 µM A23187 or LPS/fMLP was investigated. As shown in **Fig. 18A**, formation of LTB_4 and 5-H(P)ETE was inhibited concentration-dependently by **8a** and **8l** with IC_{50} values of 1.6 ± 0.3 and 1.3 ± 0.15 µM after stimulation with A23187. After priming of human whole blood with LPS for 30 min and subsequent stimulation with fMLP, **8a** reduced 5-LO product formation with an IC_{50} of 0.83 ± 0.07 µM (**Fig. 18B**).

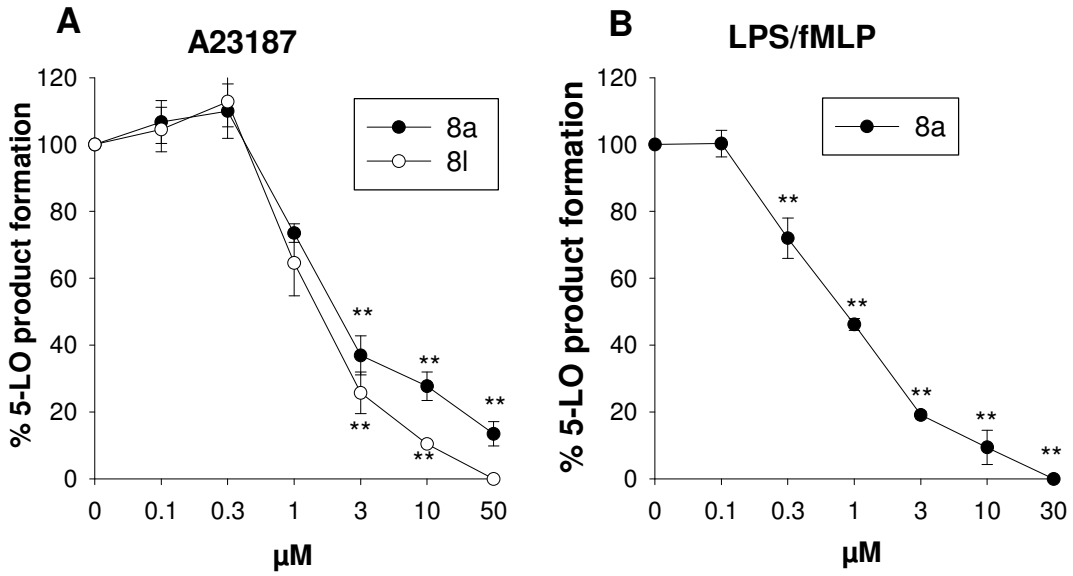


Fig. 18 5-LO inhibition in human whole blood by 8a and 8l
*Aliquots of human whole blood (1.5 ml) were pre-incubated with 8a or 8l or vehicle (DMSO 0.1%) for 10 min at 37 °C and then stimulated with 30 µM A23187 (A) or primed with LPS (1 µg/ml) for 30 min and then stimulated with 1 µM fMLP † (B). After 10 min (A23187) or 15 min (LPS/fMLP) at 37 °C, 5-LO products were analysed by HPLC. Data shown as mean ± SEM.; n = 3-4. **p< 0.01 vs. control, one-way ANOVA with post-test*

† Experiments were carried out together with Dr. C. Pergola.

4.1.2.5 *In vivo* effectiveness of 8a in carrageenan-induced pleurisy

Since the investigation in whole blood assay cannot entirely represent the in vivo characteristics of compound **8a**, it was applied in carrageenan-induced pleurisy in rats, an established model of acute inflammation involving 5-LO [240]. After i.p. or peroral administration of the test compound, pleurisy was induced by injection of a λ-carrageenan solution into the pleural cavity. 4h after pleurisy induction, exudate volume, LTB_4 levels and number of inflammatory cells were measured and compared to vehicle-treated control animals. 4 mg/kg of **8a** significantly reduced inflammatory response in the range of 10 mg/kg zileuton (**Table 7**).

***Table 7** Effect of compound 8a on carrageenan-induced pleurisy in rats*
30 min before injection of carrageenan, rats (n=10 for each experimental group) were treated i.p. with 4 mg/kg 8a, 10 mg/kg zileuton, or vehicle (DMSO 4%). Exudate volume, LTB_4 levels and accumulation of inflammatory cells in the pleural cavity were assessed 4 h after carrageenan injection. Data are expressed as mean ± S.E., n = 10.
** p<0.05; **p<0.01; *** p<0.001 vs. vehicle.*
Animal experiments were made in the group Prof. L. Sautebin, University of Naples, Italy

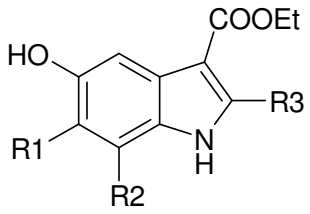
treatment	**exudate volume (ml)**	**inflammatory cells ($\times 10^6$)**	**LTB_4 (ng/rat)**
vehicle	0.48 ± 0.08	46.7 ± 3.53	1.17 ± 0.21
8a (4 mg/kg)	0.11 ± 0.0026 ***	28.0 ± 6.83 *	0.60 ± 0.096 *
zileuton (10 mg/kg)	0.11 ± 0.065 **	27.54 ± 4.41 **	0.40 ± 0.044 **

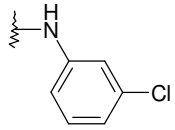
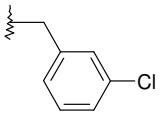
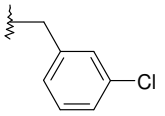
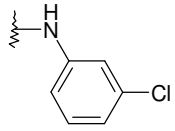
Exudate volume, number of inflammatory cells as well as the amount of LTB_4 in the pleural exudate were significantly reduced by **8a**, comparable to zileuton at 10 mg/kg. In fact, zileuton was more potent at 10 mg/kg than **8a** but still a significant decrease of LTB_4 formation could be shown for **8a**.

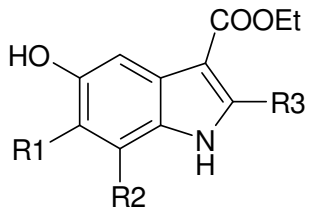
4.1.3 5-Hydroxy-indoles as dual inhibitors of 5-LO and mPGES-1

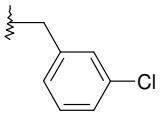
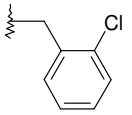
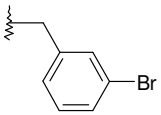
Beside assessing the compounds for 5-LO inhibition, selected derivatives were screened for inhibition of mPGES-1. Due to structural similarity with known mPGES-1 inhibitors such as MK-886, a set of hydroxy-indole-based compounds was investigated [230]. Starting from compound **1a** as lead structure, mPGES-1-derived PGE_2 production was not reduced significantly (**Table 8**).

Table 8 Inhibition of mPGES-1-derived PGE_2 formation of selected 5-hydroxy-indole-carboxylates and 5-hydroxybenzo[g]indole carboxylates [230].
Compounds were tested in microsomal preparation of A549 cells, after 15 min pre-incubation at 4 °C, 20 µM PGH_2 was added for another 1 min. and reaction was stopped and PGE_2 formation was analysed by HPLC.
n.d.: not determined



#	R 1	R2	R3	IC_{50} [µM] mPGES-1
1a	H	H		n.d.
6a	H	H		> 10
15	H	4-biphenyl		3.1
7	benzo [g]			1.6



#	R 1	R2	R3	IC_{50} [µM] mPGES-1
8a	benzo [g]			0.6
8b	benzo [g]			0.1
8f	benzo [g]			0.2

As described above for 5-LO, removing of the aminogroup in position 2 (**6a**) and enlarging the indole scaffold by anellation to benzo[g]indole-carboxylates strongly increased the potency towards mPGES-1. Introduction of a 4-biphenyl residue in position 7 (**15**) strongly enhanced the potency (IC_{50} = 3.1 µM). Besides 5-LO, compound **8a** inhibits mPGES-1 in a reversible and non-competitive manner (IC_{50} = 0.6 µM). COX-1 and COX-2 were not significantly or only barely attacked even at high concentrations in cellular assays. A more potent inhibition of mPGES-1 was found for the 2-chlorophenyl- (**8b**) and the 3-bromophenyl- substituted derivative (**8f**) with IC_{50} values of 0.1 µM and 0.2 µM, respectively.

Taken together, benzo[g]indol-hydroxyindole-3-carboxylates, exemplified by **8a** and **8l** are potent, reversible and non-competitive 5-LO inhibitors. Structural optimization of 5-hydroxy-indole carboxylates by creating benzo[g]indoles further enhanced the potency and led to dual inhibition of 5-LO and mPGES-1, whereas other enzymes of the AA cascade such as COX-1 and COX-2 were not

affected [230]. Furthermore, an *in vivo* effect was demonstrated for **8a** in an animal model of acute inflammation [239].

4.2 1,4-Benzoquinone and polyphenolic derivatives as 5-LO inhibitors

Natural sesquiterpenoids containing a 1,4-benzoquinone or hydroquinone structure such as Bolinaquinone (BLQ) or Ilimaquinone (**Fig. 19**) are present in the marine sponge species Dysidea and Smenospongia. They were ascribed to have anti-inflammatory [241], anti-microbial [242], cytotoxic and anti-proliferative properties [243].

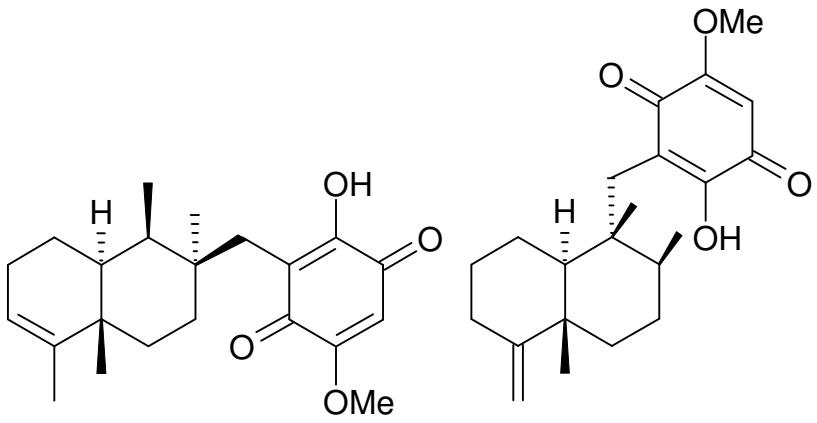


Fig. 19 Structures of the natural sesquiterpenoids Bolinaquinone (BLQ) and Illimaquinone

The anti-inflammatory properties of BLQ were demonstrated by potent inhibition of several sPLAs, but BLQ had no effect on group IV cPLA$_2$ [241]. Another study described an effect of BLQ on 5-LO activity and release of LTB$_4$ in human neutrophils with IC$_{50}$ values of 1.3 and 2.1 µM. Moreover, PGE$_2$ and NO production in zymosan-stimulated mouse peritoneal macrophages was reduced with an IC$_{50}$ of approximately 1 µM. In the mouse ear edema model, BLQ showed inhibitory effects on PGE$_2$, LTB$_4$ and TNF-α generation. Moreover, BLQ exhibited anti-inflammatory effects in several established *in vivo* models of acute and chronic inflammation [244].

The 1,4-benzoquinone structure is associated with anti-inflammatory activities in human neutrophils [245] and inhibition of PLA$_2$ preventing release of AA

[246]. In the cellular environment, the 1,4-benzoquinone structure can be reduced to hydrochinone, which may keep the iron in the catalytic centre of 5-LO in the reduced (Fe^{2+}) state, preventing catalytic activity of the enzyme.

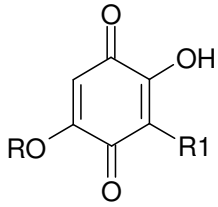
Polyphenolic compounds have often been shown to inhibit 5-LO activity, basically due to the presence of one or more hydroxygroups [190]. For the present study, compounds with 1,4-benzoquinone structures based on BLQ and a set of compounds containing polyphenolic structural elements of resorcin and veratrol were synthesized by the group of Dr. R. Filosa, University of Salerno, Fisciano, Italy.

4.2.1 SAR of benzoquinones and polyphenolic compounds

For biological investigation of the compounds two approaches were used: a cell-free assay using semi-purified recombinant 5-LO which allows to assess direct interactions with 5-LO and intact human PMNL stimulated with A23187 and exogenous AA to include effects of cell-permeability and possible influence on 5-LO activation or regulation to reduce 5-LO product formation.

BWA4C was used as control at 0.3 µM, with remaining activity of 14.7 ± 2.9% in PMNL and 56.5 ± 11.9% in purified 5-LO, respectively. For the 2-hydroxy-5-methoxy-1,4-benzoquinones (**16a,b**) and for the 2,5-dihydroxy-1,4-benzoquinone derivative **16c**, inhibition of 5-LO was only observed at high concentrations (IC_{50} > 10 µM) in both assay systems (**Table 9**)

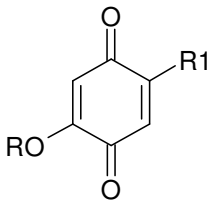
Table 9 5-LO inhibition of 2,5-dihydroxy-or 2-hydroxy-5-methoxy-benzoquinones
PMNL were stimulated with 2.5 µM A23187+20 µM AA or purified 5-LO was incubated with 1 mM $CaCl_2$ and 20 µM AA; Data shown as % of remaining 5-LO activity at 10 µM vs. DMSO control and IC_{50}; mean ± SEM, n ≥ 3
n.i.[a]: no significant inhibition up to 10 µM; n.i.[b]: no significant inhibition up to 50 µM



#	R	R1	PMNL		purified 5-LO	
			%	IC_{50} (µM)	%	IC_{50} (µM)
16a	$-CH_3$	(2-naphtyl)methyl	56.1 ± 7.4	> 10	92.3 ±16.3	43
16b	$-CH_3$	benzyl	103.3 ± 12.2	n.i.[a]	109.6 ± 15.1	n.i.[b]
16c	-H	cyclohexylmethyl	90.4 ± 12.5	n.i.[a]	95.2 ± 15.8	> 30
16d	-H	(decahydronaphthalen-2-yl) methyl	5.4 ± 2.6	0.58	51.0 ± 8.9	11

Insertion of an enlarged decahydronaphtylmethyl residue instead of cyclohexyl (**16c**) led to active compound **16d** in PMNL (IC_{50}= 0.58 µM), but only moderate potency in the cell-free asssay (IC_{50}= 11 µM). For **16d**, intact cells are obviously needed to suppress 5-LO product formation, presumably due to metabolic activation of **16d** in the cells by reduction of the benzoquinone to hydroquinone. Possibly, interference with FLAP, CLP, $cPLA_2$ or other factors regulating 5-LO such as phosphorylation, or the hydroperoxide tone can contribute to the inhibitory effect on 5-LO in intact cells but not in a cell-free system. Removal of one hydroxy group and a shift of R1 to position 2 resulted in compounds shown in **Table 10**.

Table 10 5-LO inhibition of 3-hydroxy- or 3-methoxy-benzoquinone derivatives
PMNL were stimulated with 2.5 µM A23187+ 20 µM AA or purified 5-LO was incubated with 1 mM $CaCl_2$ and 20 µM AA; Data shown as % of remaining 5-LO activity at 10 µM vs. DMSO control and IC_{50}; mean ± SEM; n ≥ 3
n.i.[a]: no significant inhibition up to 10 µM

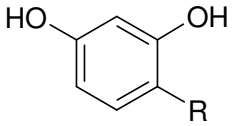


#	R	R1	PMNL		purified 5-LO	
			%	IC_{50} (µM)	%	IC_{50} (µM)
16e	-H	(2-naphtyl)methyl	5.3 ± 2.4	2.3	8.7 ± 3.7	0.78
16f	$-CH_3$	naphthalen-6-yloxy	21.2 ± 8.6	6.7	7.1 ± 3.2	0.28
16g	-H	6-carboxy-2-naphthylmethyl	74.0 ± 5.6	n.i.[a]	45.2 ± 4.0	9.0
16h	-H	4-dibenzofuranyl	74.1 ± 2.2	> 10	30.8 ± 14.9	0.8
16i	-H	benzyl	93.3 ± 16.6	n.i.[a]	74.3 ± 6.1	n.i.[a]
16k	-H	3-nitrophenyl	61.3 ± 7.8	> 10	8.0 ± 2.5	4.6
16l	-H	hexyl	15.9 ± 2.7	3.0	14.2 ± 8.6	1.4

All compounds (except **16h**) directly inhibited 5-LO. Compound **16e**, carrying a naphthalene residue, was identified as most potent compound of this series in intact neutrophils (IC_{50}= 2.3 µM). Variation of the methylene bridge between the naphtalene and the 5-hydroxy-1,4-benzoquinone core by insertion of an oxygen and subsequent methylation of the hydroxy group at position 5 (**16f**) decreased the potency almost 3-fold in the cell-based test system, whereas the potency of **16f** was increased in the cell-free assay to an IC_{50} of 0.28 µM. Introduction of a carboxy group at position 6 of the naphthalene ring (**16g**) was clearly detrimental compared to **16e**, leading to a more than 30-fold higher IC_{50} value in cell-free assay and no inhibition in PMNL. A larger heterocyclic

substituent such as dibenzofurane (**16h**) restored the potency in the cell-free assay with an IC_{50} of 0.8 µM comparable to **16e**. Interestingly, the replacement of the naphtalene moiety by benzyl (**16i**) completely abrogated 5-LO inhibition. The direct connection of the benzoquinone core with 3-nitrophenyl (**16k**) led to weak potency in intact cells, but some bioactivity was retained in the cell-free assay. The nitro group of **16k** is probably reduced in the cell to an amino group leading to loss of inhibition in PMNL. The weak inhbition of **16g-16k** in PMNL is explainable by a hindered cellular uptake (**16h, i**) or metabolic reactions (**16g, k**) leading to inactivation in the cells. When an aliphatic side chain (**16l**) was present at the hydroxy-benzoquinone, the potency was in a comparable range to **16e** (IC_{50}= 3.0 µM in PMNL and 1.4 µM in the cell-free assay, respectively). In addition to the benzoquinones, various polyphenolic derivatives based on resorcin (**Table 11**) as well as catechol and veratrol (**Table 12**) were synthesized and evaluated for 5-LO inhibition.

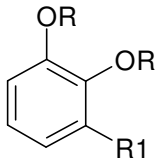
Table 11 5-LO inhibition of resorcin derivatives
PMNL were stimulated with 2.5 µM A23187+20 µM AA or purified 5-LO was incubated with 1 mM $CaCl_2$ and 20 µM AA; Data shown as % of remaining 5-LO activity at 10 µM vs. DMSO control and IC_{50}; mean ± SEM; n ≥ 3
n.i .[a]: no significant inhibition up to 10 µM



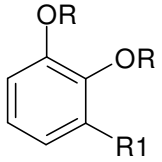
#	R	PMNL		purified 5-LO	
		%	IC_{50} (µM)	%	IC_{50} (µM)
17a	(2-naphtyl)methyl	8.9 ± 1.0	2.3	37.2 ± 4.9	7.4
17b	4-dibenzofuranyl	84.1 ± 12.6	n.i.[a]	30.7 ± 8.8	6.3
17c	benzyl	60.0 ± 2.2	> 10	47.6 ± 4.4	9.4
17d	phenyl	22.7 ± 5.3	4.2	9.4 ± 2.4	2.8
17e	3-nitrophenyl	64.1 ± 7.4	> 10	84.3 ± 8.2	> 10
17f	hexyl	18.6 ± 6.1	2.8	25.6 ± 7.1	4.7

Combination of the resorcinol structure with aromatic residues such as naphylmethyl (**17a**), dibenzofuranyl (**17b**) or phenyl (**17d**) yielded compounds with moderate potencies in the low micromolar range in the cell-free assay. However, enlargement of the aromatic system to dibenzofuranyl (**17b**) abolished the inhibitory effect in intact cells as observed before for the quinone series. Variation of the distance between the resorcinol core and the substituent by introduction of by a methylene spacer (**17c**) as well as insertion of a nitro group at the phenyl ring (**17e**) was detrimental compared to **17d**. Replacement of the aromatic structures by an aliphatic hexyl chain (**17f**) yielded an active compound, but did not further enhance the potency in both read outs. Within the catechol series (**Table 12**), **18a** and **18b** emerged as most active catechol derivatives in the cell-free assay with IC_{50} values of 130 nM and 76 nM, respectively. However, **18a**, carrying a 4-nitrophenyl residue, was completely inactive in the cell-based assay, probably due to metabolic conversion of the nitro group which does not occur under the conditions of the cell-free assay.

Table 12 5-LO inhibition of catechol (R=H) and veratrol (R=CH_3) derivatives
PMNL were stimulated with 2.5 µM A23187 +20 µM AA or purified 5-LO was incubated with 1 mM $CaCl_2$ and 20 µM AA; Data shown as % of remaining 5-LO activity at 10 µM vs. DMSO control and IC_{50}; mean ± SEM; n ≥ 3,
n.i. [a]: no significant inhibition up to 10 µM



#	R	R1	PMNL		purified 5-LO	
			%	IC_{50} (µM)	%	IC_{50} (µM)
18a	-H	4-nitrophenyl	132.7 ± 42.4	n.i.a	0 ± 0	0.13
18b	-H	4-dibenzofuranyl	2.7 ± 1.2	0.6	0 ± 0	0.076
19a	$-CH_3$	4-nitrophenyl	66.5 ± 7.9	> 10	94.9 ± 5.9	n.i.[a]
19b	$-CH_3$	naphtyl-6-oxymethyl	21.7 ± 5.0	2.9	84.2 ± 5.5	n.i.[a]



#	R	R1	PMNL		purified 5-LO	
			%	IC_{50} (µM)	%	IC_{50} (µM)
19c	$-CH_3$	naphtylmethyl	35.4 ± 5.3	5.2	87.3 ± 6.5	n.i.[a]

In contrast to the 1,4-benzoquinones and resorcinol derivatives, substitution of the catechol core with dibenzofuranyl (**18b**) was advantageous leading to the most potent representative with IC_{50} values of 600 nM and 76 nM in PMNL and the cell-free-assay, respectively. Reducing conditions in the cell lead to inactivation of **18a** due to reduction of the nitrophenyl group to an aniline group, since **18a** is completely inactive in the cell-based assay, whereas purified 5-LO was potently inhibited with an IC_{50} of 0.13 µM. Thus, **18b** is apparently not influenced by reducing conditions in the cell and acts as 5-LO inhibitor. In contrast to the catechol compounds, the veratrol analogues **19a-c** were completely inactive in the cell-free assay, showing that the hydroxy groups of **18a** play an important role for 5-LO inhibition compared to **19a**.

Variation of the assay conditions by pre-incubation of PMNL with the veratrol derivatives (**19a-c**) up to 60 min did not influence the potency. This suggests that metabolic changes such as potential cleavage of the methoxy group have no influence on the inhibitory action. This approach also excluded cytotoxic effects of the compounds up to 10 µM, since after 60 min pre-incubation time, 5-LO product formation in PMNL was the same as after 15 min pre-incubation time with the compounds (data not shown).

4.2.2 Pharmacological characterization of selected compounds

4.2.2.1 Investigation of scavenging properties

Since polyphenols and benzoquinones are assumed to act via their redox-activity, selected compounds were assessed in the DPPH assay which

determines radical scavenging and reducing properties of a compound. This assay investigates the ability of a compound to reduce the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical to diphenylpicryl-hydrazine. Ascorbic acid and cysteine were used as positive controls, since DPPH reacts in a 1:1 stoichiometry with cysteine to cystine, whereas for oxidation of ascorbic acid 2 mol DPPH are needed. When DPPH is reduced by the test compound, the colour change from violet to transparent is measured photometrically at 520 nm [247]. Weak reducing properties were found for the 3-hydroxybenzochinone **16l**, since only one oxidable hydroxy group is present in the molecule, whereas the polyphenolic catechol derivatives **18a** and **18b** showed strong reducing activities equipotent to ascorbic acid (**Fig. 20A**).

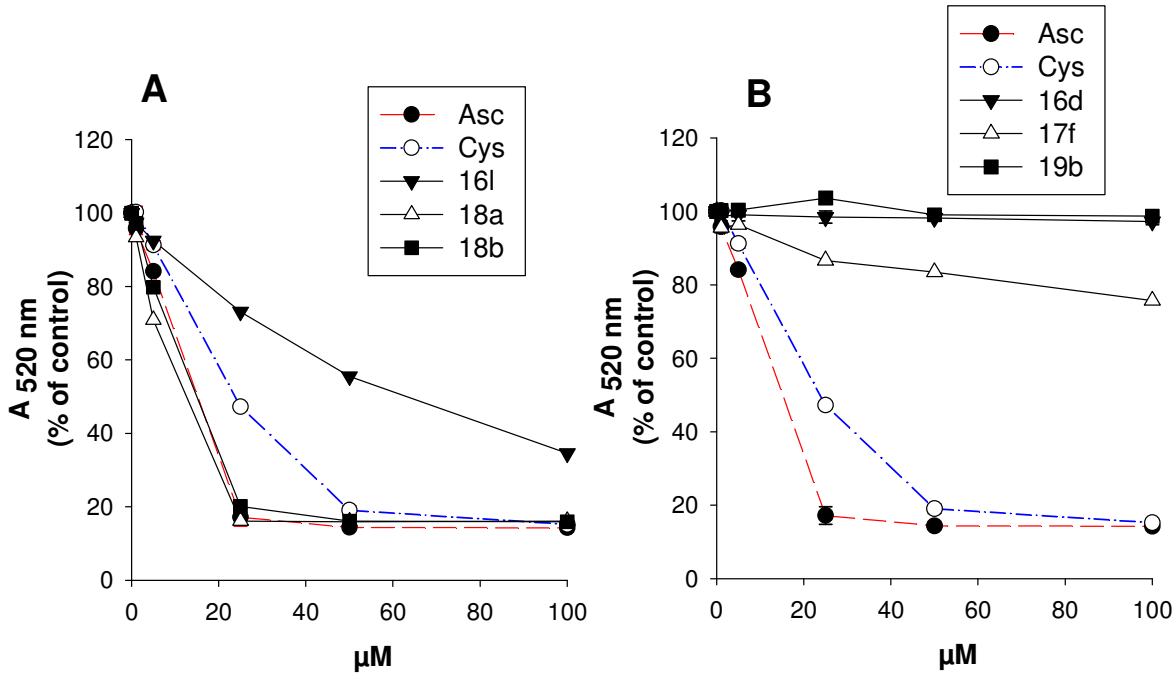


Fig. 20 Radical scavenging activities of benzoquinones and polyphenols (DPPH assay)
Ethanolic solutions of test compounds were incubated for 30 min at RT with acetate-buffered solution of 100 µM DPPH in ethanol. Extinction at 520 nm was measured and DMSO (2%) sample was set as 100 %. Data shown are representative for one experiment out of up to three experiments. Asc=ascorbic acid; Cys=Cysteine

Interestingly, the 2,5-dihydroxybenzoquinone derivative **16d** showed no reducing properties up to 100 µM (**Fig. 20B**). Due to stabilisation of the dihydroxybenzoquinone structure, its vinylogic acid structure and formation of

hydrogen bonds between the proximate keto and hydroxy groups, **16d** did not reduce DPPH under these conditions. No reducing properties were found, neither for **17f** due to energetically disadvantageous 1,3-positioning of the hydroxy groups. As expected, the veratrol derivative **19b**, carrying non-oxidizable methoxy groups could not reduce the DPPH reagent in this assay.

To sum up, the polyphenolic compounds and benzoquinone derivatives are potent inhibitors of 5-LO *in vitro*. Since not all compounds have direct reducing properties, their inhibitory action is achieved following metabolic activation in the cell and might not only be ascribed to their polyphenolic or quinone structure.

4.2.2.2 Inhibition of 12- and 15-LOs

Next, the selectivity of the compounds towards 12-and 15-LOs was analysed in PMNL. Inhibition of 12-HETE formation, formed by oxidation of AA by p12-LO and 15-HETE, formed by 15-LO were analysed for selected compounds in PMNL (**Fig. 21**).

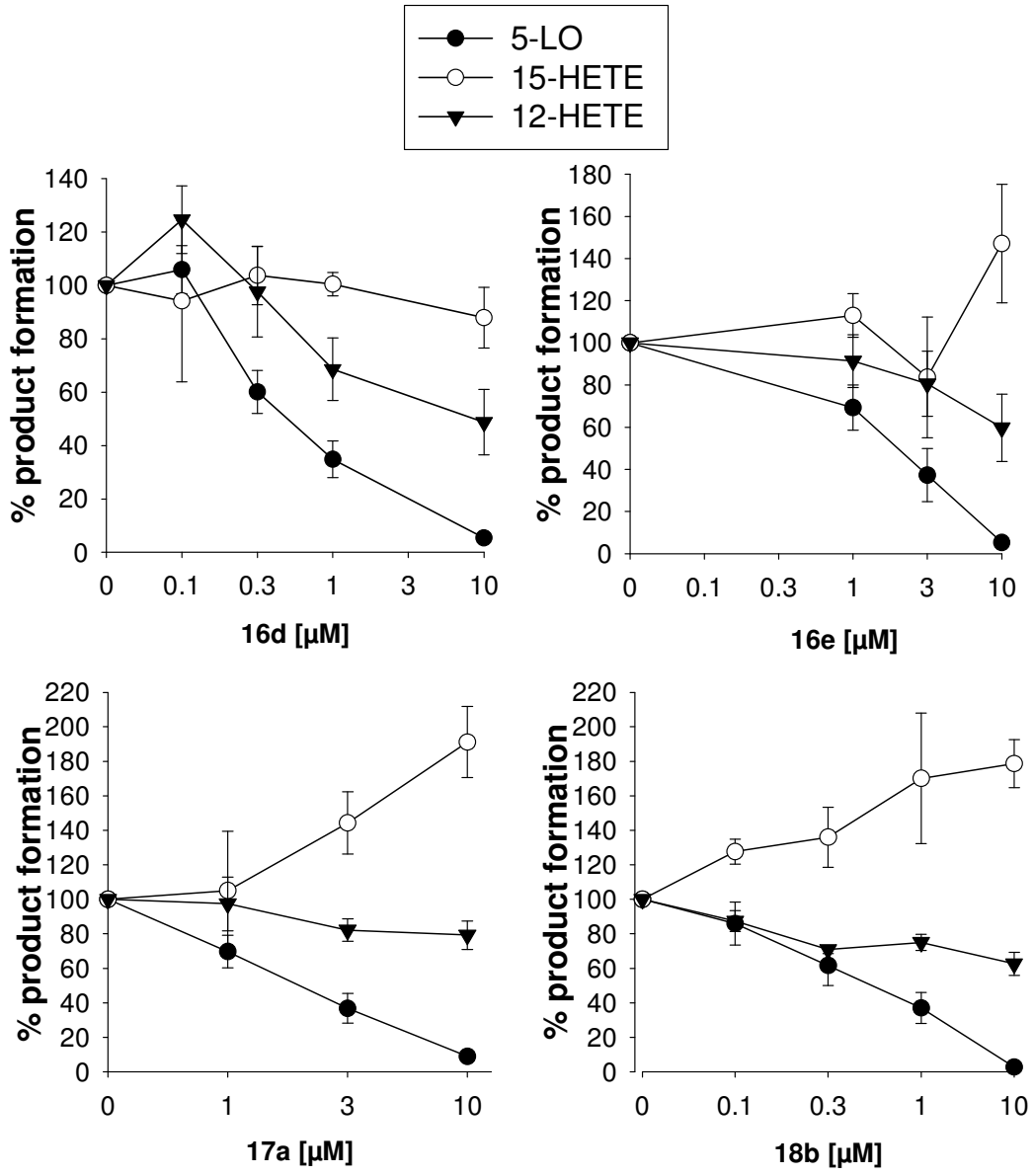


Fig. 21 Effects of selected compounds on p12-LO and 15-LO product formation
PMNL were pre-incubated for 15 min at 37 °C. Then, 2.5 µM A23187 + 20 µM AA was added. After 10 min at 37 °C, 12- and 15-HETE formation was analysed by HPLC. 5-LO products include LTB_4, its trans-isomers and 5-HETE. Data shown as mean ± SEM; n= 2-3

For all compounds, 15-HETE formation was not influenced or rather increased concentration-dependently. This effect might be interpreted as a shunt of AA to 15-LO in neutrophils and subsequent product formation. 12-HETE formation was diminished by the hydroxybenzoquinones **16d** and **16e** with IC_{50} values greater than 10 µM. The resorcinol derivative **17a** and the catechol compound

18b inhibited 5-LO selectively without influence on 12-HETE formation up to 10 µM.

4.2.2.3 Inhibition of 5-LO in human whole blood

In order to describe the *in vivo* behaviour of selected compounds, 5-LO inhibition in A23187-stimulated human whole blood was assessed. 5-LO products were hardly reduced between 8 and 22% by 10 µM of **16e**, **17a** and **17f** (data not shown). On the other hand, **16d** and **18b** reduced 5-LO product formation in a concentration-dependent manner with IC_{50} values of approximately 9 µM (**Fig. 22**).

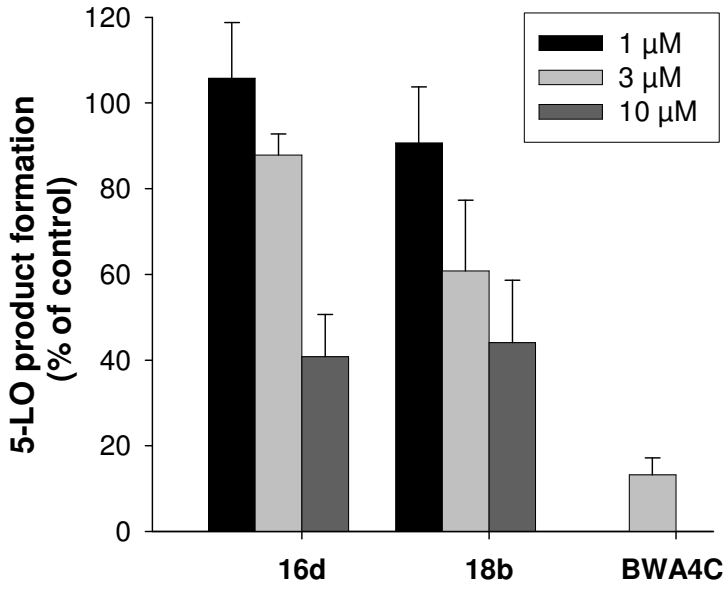


Fig. 22 5-LO inhibition of 16d and 18b in human whole blood
Aliquots of human whole blood (1.5 ml) were pre-incubated with the compounds for 10 min at 37 °C and then stimulated with 30 µM A23187. After 10 min at 37 °C, 5-LO products were analysed by HPLC. Data shown as mean ± SEM; n=2-3

3 µM BWA4C was used as positive control that inhibited 5-LO product formation in whole blood as expected [191]. Unfortunately, no statistical significance of 5-LO inhibition was evident, presumably due to the low sample size. Priming of human whole blood with LPS following stimulation with the chemotactic peptide fMLP resulted in an IC_{50} of 4.1 ± 0.6 µM for **16d** [248].

4.2.2.4 Molecular docking studies

In order to identify the exact binding mode, interactions of the molecules and amino acids in the active site of 5-LO were studied by docking experiments. Molecular docking experiments were performed in the group of Prof. P. Reddanna at University of Hyderabad, India.

The free energy of binding during the docking procedure was chosen to describe the affinity of the ligands to the active site of 5-LO and compared with the experimental IC_{50} values obtained in the *in vitro* experiments. A good correlation was found between the inhibition in the cell-free assay and the free binding energy. The molecular docking experiments predicted the 1,4-benzoquinones to be more active than the resorcinol derivatives, correlating to the potency in the cell-free assay [249].

4.2.2.5 *In vivo* experiments

Two well-established animal models of inflammation were applied for further characterization of the anti-inflammatory effectiveness of **16d**, the carrageenan-induced mouse paw edema and the mouse air pouch model. All animal experiments were conducted at University of Naples, Italy [248]. When **16d** was given i.p. at 0.1-10 mg/kg 30 minutes prior to intraplantar injection of carrageenan hind paw swelling was significantly reduced over the whole time period of 72 h with maximal effects at a dose of 1 mg/kg. **16d** was also investigated in the air pouch model, where sterile air is injected subcutaneously several times into the back of mice followed by administration of zymosan or another inflammatory stimulus. **16d** significantly reduced zymosan-induced cell migration in the exudate at 0.1 mg/kg after i.p. administration 30 minutes prior to zymosan [248]

In summary, this chapter decribes the discovery of a series of 1,4-benzoquinones and polyphenolic compounds as inhibitors of 5-LO. SAR studies revealed that the substitution pattern strongly determines the potency of the compounds. Docking experiments demonstrated a direct binding of selected compounds to 5-

LO, which correlated to the results from the *in vitro* experiments with recombinant 5-LO. Further investigations of the 1,4-benzoquinone compound **16d** confirmed its good anti-inflammatory efficiency in two *in vivo* animal models.

4.3 Derivatives of pyrazole-3-propanoic acid as 5-LO inhibitors

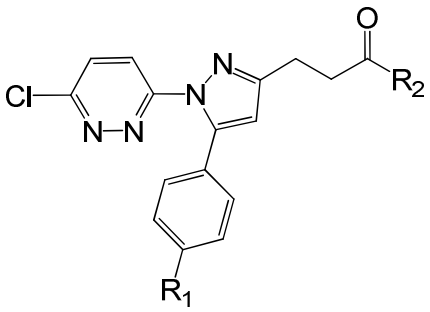
1,5-Diarylpyrazol-3-propanoic acids were recently reported as inhibitors of COX-1, COX-2 or 5-LO [250]. In order to improve the inhibition of LT formation, esters and amide derivatives of 1,5-diarylpyrazol-3-propanoic acid were synthesized by the group of Prof. E. Banoglu at the University of Ankara, Turkey and biologically evaluated for their effects on 5-LO product formation in our lab. A cell-based assay using human neutrophils was applied to demonstrate effects resulting in reduced LT formation apart from direct inhibition of 5-LO. Additionally, cell-free assays using recombinant human 5-LO from homogenates of E.coli (S40) or further purified 5-LO by ATP-affinity chromatography (semi-purified 5-LO) were carried out.

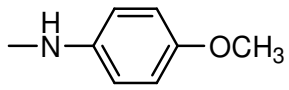
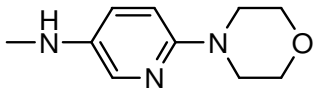
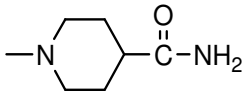
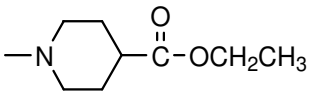
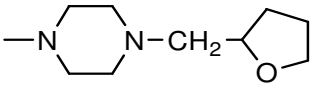
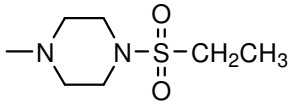
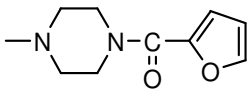
4.3.1 SAR of pyrazole-3-propanoic acid derivatives

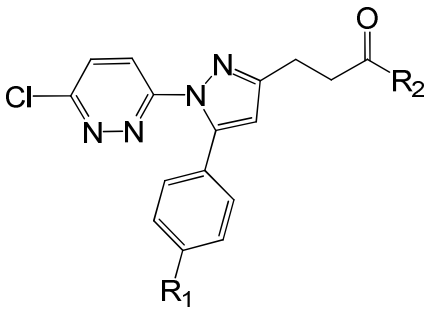
The first set comprised 18 derivatives based on 3-(1-(6-chloropyridazin-3-yl)-5-phenyl-1H-pyrazol-3-yl)propanoic acid. The compounds were investigated for 5-LO inhibition at a concentration of 10 μM in isolated human neutrophils (PMNL) and supernatants of E.coli lysates expressing human recombinant 5-LO (S40). PMNL were activated by 2.5 μM Ca^{2+}-ionophore A23187 to elevate intracellular calcium concentration and 20 μM AA to circumvent inhibition at the stage of substrate supply by $cPLA_2$ (**Table 13**).

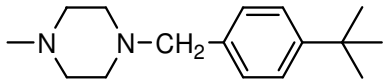
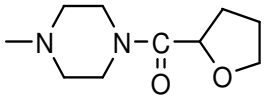
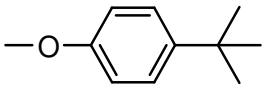
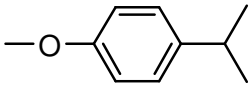
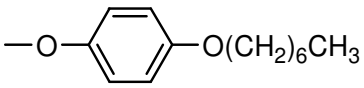
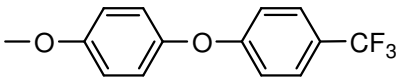
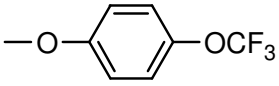
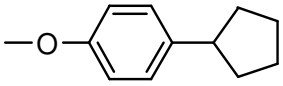
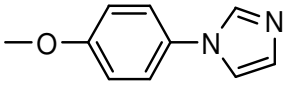
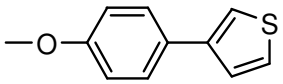
Table 13 Inhibition of 5-LO product formation by pyridazinyl-substituted-pyrazole-3-propanoic acid derivatives

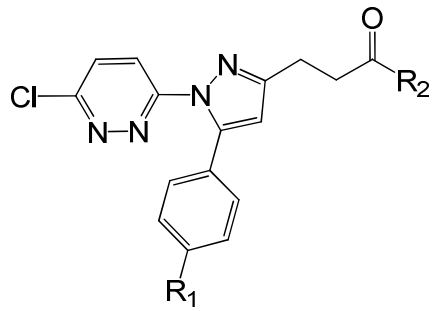
PMNL were stimulated with 2.5 µM A23187+20 µM AA or aliquots of 40,000 x g E. coli homogenates expressing recombinant 5-LO (S40) were incubated with 2 mM $CaCl_2$ and 20 µM AA; Data shown as % of remaining 5-LO activity at 10 µM (mean ± SEM) versus vehicle (0.3% DMSO and IC_{50} if available), n=3;
n.d.: not determined

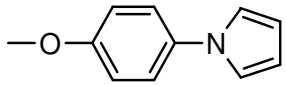


#	R1	R2	PMNL % at 10 µM	S40 % at 10 µM	IC_{50} PMNL [µM]
26	-F		88.3 ± 7.7	91.6 ± 14.9	n.d
27	$-CH_3$		106.2 ± 6.4	90.0 ± 11.0	n.d
28	$-CF_3$		104.3 ± 6.3	102.9 ± 17.3	n.d
29	$-CF_3$		62.0 ± 7.9	72.6 ± 9.2	15
30	$-CH_3$		86.0 ± 11.5	105.6 ± 16.4	n.d
31	$-CH_3$		96.3 ± 5.0	93.6 ± 6.9	n.d
32	$-CF_3$		81.8 ± 10.1	125.5 ± 22.7	n.d



#	R1	R2	PMNL % at 10 µM	S40 % at 10 µM	IC_{50} PMNL [µM]
33	-F		23.4 ± 4.6	55.3 ± 10.5	1.8
34	-F		95.0 ± 5.4	90.8 ± 9.6	n.d
35	-F		67.3 ± 15.8	92.7 ± 4.3	n.d
36	$-CH_3$		46.4 ± 9.3	92.7 ± 12.7	8
37	-F		90.1 ± 7.7	86.5 ± 8.7	n.d
38	$-CH_3$		86.5 ± 4.2	95.0 ± 10.9	n.d
39	$-CH_3$		70.5 ± 12.3	115.8 ± 6.5	n.d
40	$-CF_3$		77.2 ± 6.9	92.0 ± 6.3	n.d
41	-F		111.4 ± 4.4	71.7 ± 10.9	n.d
42	-F		90.8 ± 6.6	83.4 ± 24.9	n.d



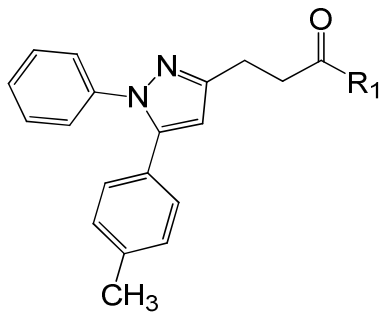
#	R1	R2	PMNL % at 10 µM	S40 % at 10 µM	IC_{50} PMNL [µM]
43	$-CF_3$		94.1 ± 11.3	90.8 ± 14.9	n.d

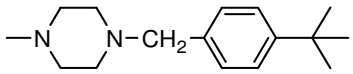
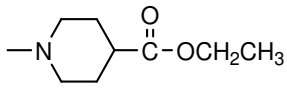
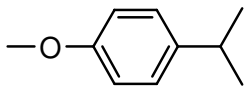
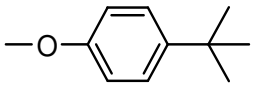
Four compounds within this series inhibited 5-LO product formation remarkably in the cell-based assay at 10 µM. Two piperazine analogs substituted with ethyl piperidinecarboxylate (**29**) and tert-butylbenzylpiperazine (**33**) inhibited LT formation at 10 µM leading to 62% and 23% of remaining 5-LO activity in the cell-based asssay, respectively. Derivatization of pyrazole-3-propanoic acid with primary amines such as 4-methoxyaniline (**26**) or 6-morpholino-pyridine-3-amine (**27**) led to inactive compounds. Linkage of the carboxylic group with piperidinecarboxamide (**28**) was also not successful, but with more lipophilic ethyl piperidinecarboxylate (**29**) the IC_{50} in PMNL was raised to 15 µM. When piperazine was linked with the carboxylic group, only the lipophilic bulky tert-butylbenzyl substituent (**33**) was tolerated, whereas the other more polar piperazine analogs (**30-32, 34**) were not active. For the esterified compounds, a remarkable decrease of 5-LO product formation in PMNL was observed only for the tert-butylphenoxy (**35**) and isopropylphenoxy (**36**) substituents, whereof **36** was more potent with an IC_{50} of 8 µM in PMNL. Variations at the phenolic moiety involved introduction of heptyloxy (**37**), trifluoromethylphenoxy (**38**), trifluoromethoxy (**39**) or cyclopentyl (**40**) substituents with poor outcome. In general, heterocycles such as imidazole (**41**), thiophene (**42**) or pyrrole (**43**) seem to diminish the potency of the compounds. In the cell-free assay, an IC_{50} of approximately 10 µM was observed for compound **33**, suggesting rather

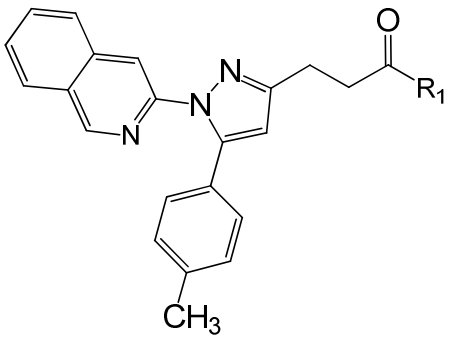
influence on the cellular regulation of 5-LO. None of the other compounds showed remarkable 5-LO inhibition in the cell-free assay.

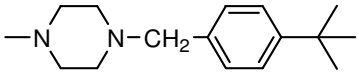
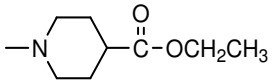
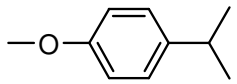
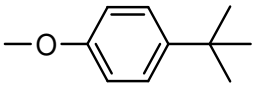
A second series of compounds was designed and synthesized based on the identified active compounds (**29, 33, 35, 36**) from the first set. Simultaneously, modifications at the pyrazole core were made by exchange of the chloropyridazinyl residue at N-1 by phenyl (**44-47**) or isoquinoline moieties (**48-51**) (**Table 14**). It has to be noted that stimulation of the PMNL was done with 2.5 µM A23187 without exogenous AA in order to identify inhibitors of FLAP.

Table 14 Inhibition of 5-LO product formation by pyrazole-propanoic acid derivatives
PMNL were stimulated with 2.5 µM A23187 or aliquots of E. coli homogenates expressing recombinant 5-LO (S40) were incubated with 2 mM $CaCl_2$ and 20 µM AA; Data shown as % of remaining 5-LO activity at 10 µM (mean ± SEM) versus vehicle (0.3% DMSO); n ≥ 3



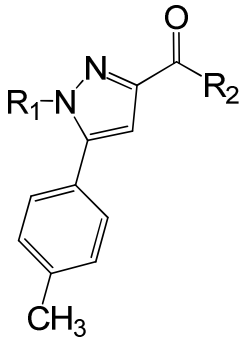
#	R1	PMNL % at 10 µM	S40 % at 10 µM
44		30.5 ± 7.4	58.9 ± 11.1
45		7.2 ± 3.5	102.5 ± 11.6
46		6.2 ± 2.5	95.3 ± 6.2
47		107.6 ± 19.2	80.7 ± 1.8

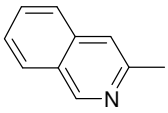
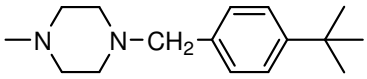
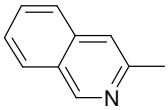
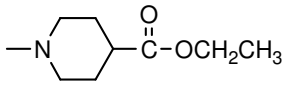
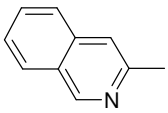
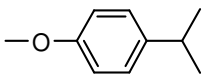
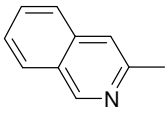
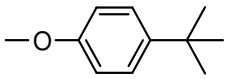
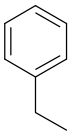
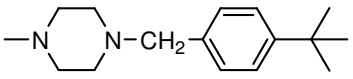
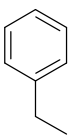
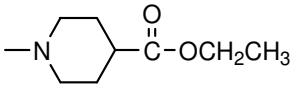


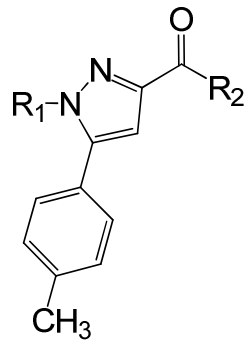
#	R1	PMNL % at 10 µM	S40 % at 10 µM
48		68.3 ± 2.1	51.5 ± 6.1
49		65.8 ± 12.7	69.9 ± 9.9
50		40.1 ± 7.8	79.6 ± 12.3
51		n.a. (*compound insoluble)*	

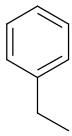
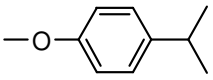
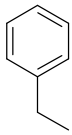
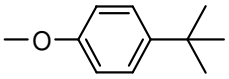
Compounds **44-46**, carrying a phenyl residue at N-1, as well as the isoquinoline-substituted derivative **50**, reduced 5-LO product formation significantly in PMNL at 10 µM. Again, no or only moderate inhibition (**44** and **48**) in the cell-free assay was observed at 10 µM. Further modifications involved shortening of the side chain at C-3 of the pyrazole. In comparison to the isoquinoline-substituted compounds **48-51**, potency was impaired for the isoquinoline derivatives **52-55** (**Table 15**), suggesting that the propanoic acid structure is superior to direct connection of R2 with the pyrazole.

Table 15 Inhibition of 5-LO product formation by N1-substituted pyrazole derivatives
PMNL were stimulated with 2.5 µM A23187 or aliquots of E. coli homogenates expressing recombinant 5-LO (S40) were incubated with 2 mM $CaCl_2$ and 20 µM AA;
Data shown as % of remaining 5-LO activity at 10 µM (mean ± SEM) versus vehicle (0.3% DMSO), n ≥ 3



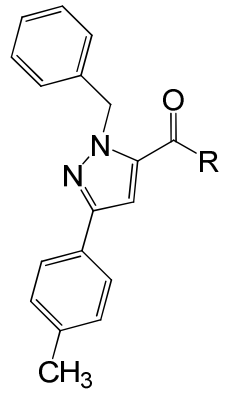
#	R1	R2	PMNL % at 10 µM	S40 % at 10 µM
52			80.7 ± 21.3	75.4 ± 15.1
53			75.8 ± 7.8	74.8 ± 10.0
54			85.8 ± 4.0	91.4 ± 7.7
55			92.7 ± 8.0	89.5 ± 10.9
56			59.4 ± 7.2	76.8 ± 5.1
57			44.7 ± 7.2	68.8 ± 5.9

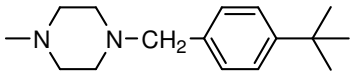
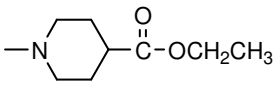
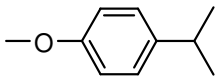
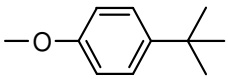


#	R1	R2	PMNL % at 10 µM	S40 % at 10 µM
58			97.1 ± 8.9	87.7 ± 6.0
59			94.5 ± 10.5	98.2 ± 12.8

Further structural modifications by addition of benzyl (**56-59**) or repositioning of the benzyl to N-2 of the pyrazole (**60-63**) failed to increase the potency (**Table 16**). BWA4C was used as control inhibitor at a concentration of 0.3 µM leading to 13.0 ± 3.8% remaining 5-LO activity in PMNL and 31.5 ± 7.9% in the cell-free assay, respectively.

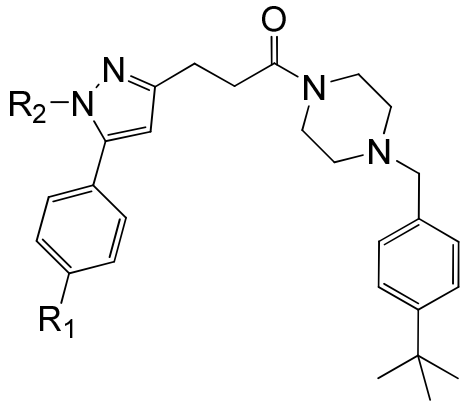
Table 16 inhibition of 5-LO product formation by benzyl-substituted pyrazole derivatives
PMNL were stimulated with 2.5 µM A23187 or aliquots of E. coli homogenates expressing recombinant 5-LO (S40) were incubated with 2 mM $CaCl_2$ and 20 µM AA;
Data shown as % of remaining 5-LO activity at 10 µM (mean ± SEM) versus vehicle (0.3% DMSO); n ≥ 3

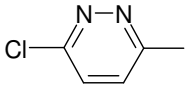
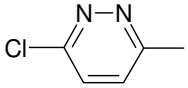
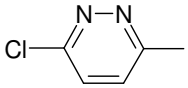
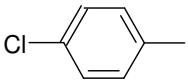
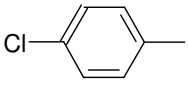
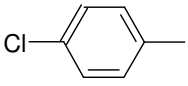
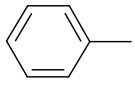


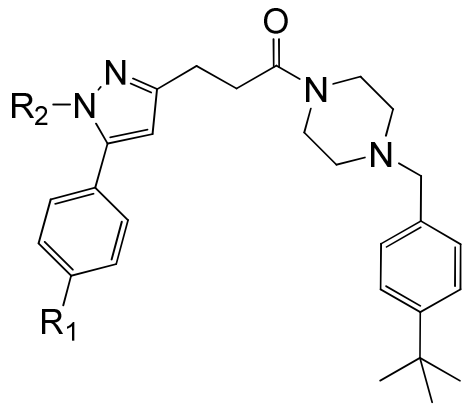
#	R	PMNL % at 10 µM	S40 % at 10 µM
60		107.0 ± 12.2	92.6 ± 5.6
61		90.6 ± 2.8	72.1 ± 12.0
62		105.1 ± 9.7	113.7 ± 5.9
63		103.0 ± 4.6	102.7 ± 11.3

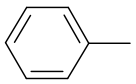
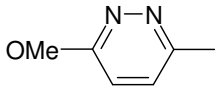
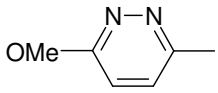
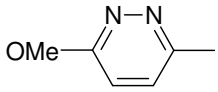
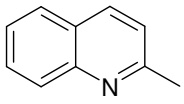
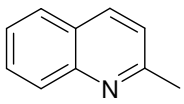
In the next step, compound **33** was subject of further structural modifications. Variations of the p-substituent on the phenyl ring at C-5 of pyrazole by trifluoromethyl, methyl or flourine and replacement of chloropyridazine at N-1 of the pyrazole (**33,64,65**) by aromatic moieties such as 4-chlorophenyl (**66-68**), phenyl (**69,70**), 6-methoxypyridazin-3-yl (**71-73**) or quinolin-2-yl (**74,75**) led to compounds listed in **Table 17.**

Table 17 Inhibition of 5-LO product formation by derivatives based on compound 33
PMNL were stimulated with 2.5 µM A23187 +20 µM AA or aliquots of E. coli homogenates expressing recombinant 5-LO (S40) were incubated with 2 mM $CaCl_2$ and 20 µM AA;
Data shown as % of remaining 5-LO activity at 10 µM (mean ± SEM) versus vehicle (0.3% DMSO); n=2-3



#	R1	R2	PMNL % at 10 µM	purified 5-LO % at 10 µM	IC_{50} [µM] PMNL
33	-F		23.4 ± 4.6	102.8 ± 11.2	1.8
64	$-CF_3$		17.4 ± 8.1	88.8 ± 28.4	2.9
65	$-CH_3$		14.6 ± 0.5	107.4 ± 25.0	2.8
66	-F		13.2 ± 3.4	143.3 ± 3.5	2.6
67	$-CF_3$		43.9 ± 9.9	137.8 ± 37.5	8.0
68	$-CH_3$		27.7 ± 8.4	111.3 ± 23.6	2.9
69	-F		3.7 ± 2.2	78.5 ± 20.4	2.5



#	R1	R2	PMNL % at 10 µM	purified 5-LO % at 10 µM	IC_{50} [µM] PMNL
70	$-CF_3$		12.7 ± 4.4	109.3 ± 25.2	2.9
71	-F		50.1 ± 14.1	69.2 ± 3.5	> 10
72	$-CF_3$		36.2 ± 15.4	56.1 ± 21.8	8
73	$-CH_3$		27.1 ± 4.6	86.1 ± 8.2	3.3
74	-F		10.4 ± 5.2	114.1 ± 31.6	2.8
75	$-CF_3$		44.5 ± 16.3	87.3 ± 24.8	3

Introduction of chlorophenyl (**66-68**) and quinoline (**74,75**) instead of chloropyridazine retained potency with IC_{50} values in the range of 1.8 µM (**33**) and 3 µM (**75**). For **67**, trifluoromethyl substitution at p-position of C5-phenyl was detrimental leading to almost 3-fold loss of potency compared to the corresponding chloropyridazine compound **64**. Replacement with phenyl residues (**69, 70**) as seen before for compound **44** and variation of the p-position of the phenyl ring kept potency in the range of the corresponding

chloropyridazine derivatives. However, introduction of methoxypyridaztine residues (**71-73**) at the central pyrazole core was disadvantageous, especially together with fluoro- or trifluormethyl substitution at the para-position of the phenyl ring at C-5. As described for the derivatives before, recombinant semi-purified 5-LO was not remarkably inhibited at concentrations up to 10 µM.

4.3.2 Pharmacological characterization of selected compounds

In order to study the influence of the substitution patterns at C-5 and N-1 of the pyrazole core on the mode of action, 9 compounds were chosen and subjected to further investigations (**Fig. 23**). For this purpose, the chloro-pyridazine-substituted derivatives **29**, **33**, **36** and the respective phenyl- and isoquinoline-substituted derivatives (**44-46** and **48-50**) were chosen for further experiments.

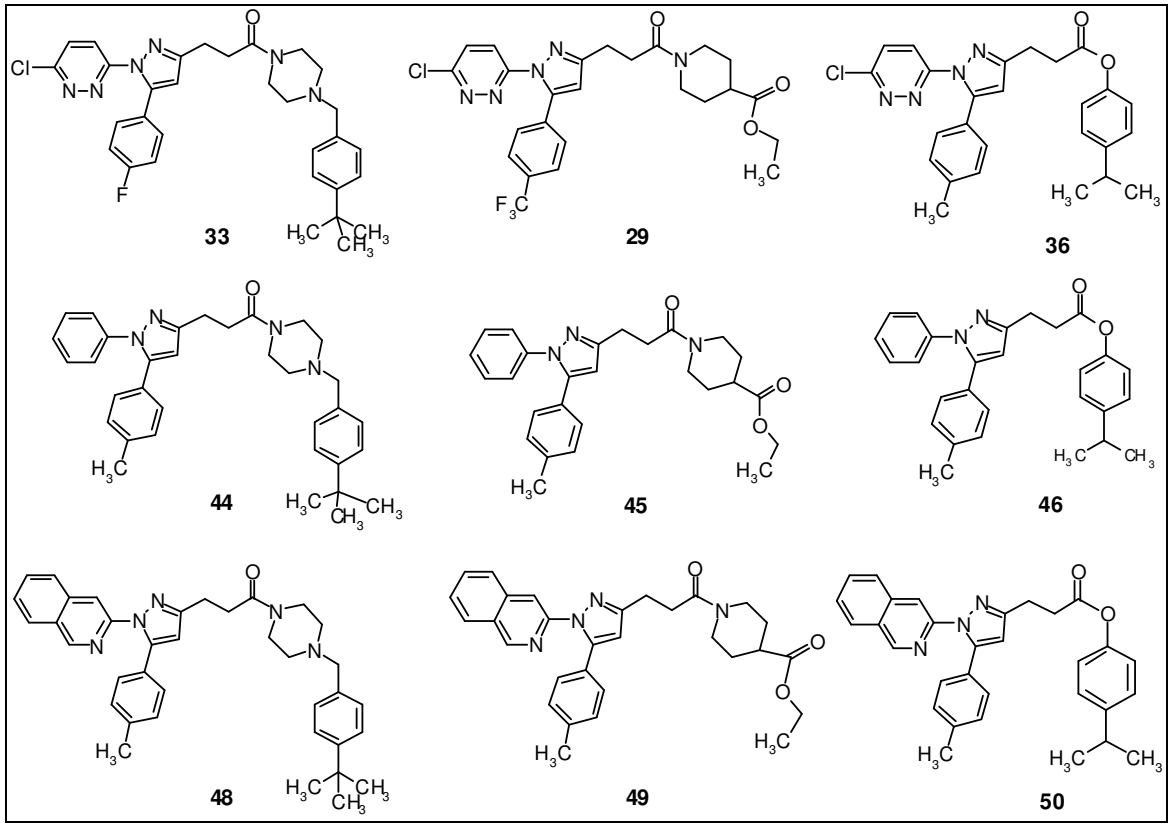


Fig. 23 Selected compounds for pharmacological characterization

For example, the influence of the substrate concentration, alternative stimulation of the cells, selectivity towards other LOs, COXs and mPGES-1 or influence on

the regulation of 5-LO were investigated to characterize the inhibitory actions of the compounds.

4.3.2.1 Influence of AA concentration in PMNL

The substrate concentration is an important parameter to identify a possible competitive mode of action. Accordingly, PMNL were stimulated with calcium ionophore with or without AA at different concentrations (**Fig. 24**).

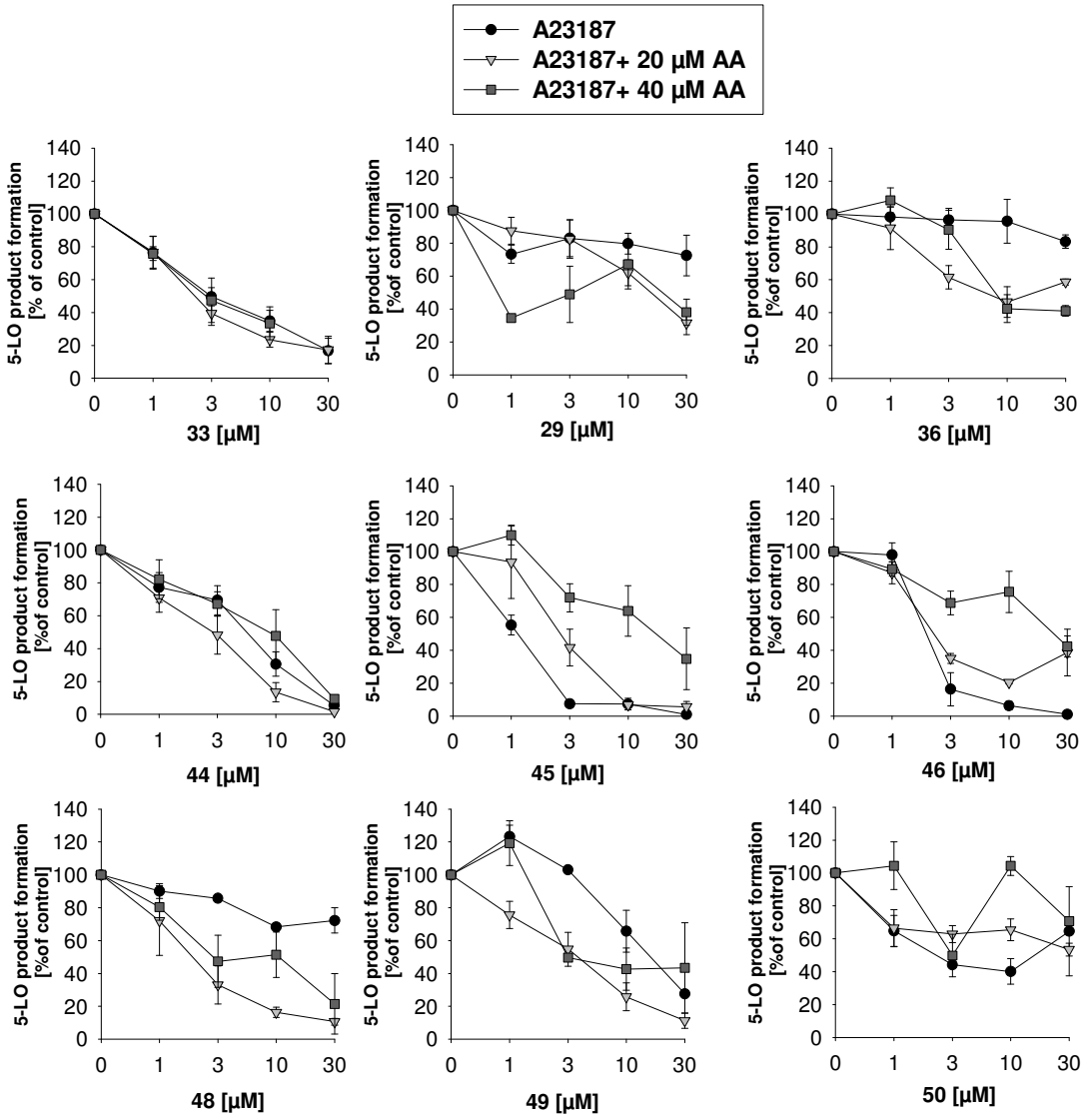


Fig. 24 Efficiency of the compounds at different substrate concentrations
PMNL (5 x 10^6/ml with exogenous AA) or 1 x10^7/ml (without exogenous AA) were pre-incubated for 15 min at 37 °C with the compounds or DMSO. Then, 2.5 µM A23187 was added together with or without the indicated amounts of AA. After 10 min at 37 °C, 5-LO products were analysed by HPLC. Data shown as mean ± SEM; n=3-8

For the chloro-pyridazine-substituted derivative **33**, no significant influence of substrate concentration on the efficacy was observed. Stimulation of the cells with A23187 together with 20 µM AA resulted in an IC_{50} of 1.8 µM. Only a small shift to approximately 3 µM occurred after stimulation with A23187 as well as together with 40 µM AA. Interestingly, the derivatives **29**, **36** and **48** inhibited 5-LO only in presence of exogenous AA. The IC_{50} of **29** was about 15 µM and 10 µM for **36** when the cells were stimulated with 20 µM or 40 µM of AA. The reduced LT formation only in presence of exogenous AA might be caused by formation of vesicles consisting of AA and the test compound resulting in higher reduction of product formation *in vitro*. While for **44** a competitive mode of action seems not reasonable, **45** and **46** were most potent upon stimulation without exogenous AA. For both, the efficiency was strongly impaired when exogenous AA was added together with A23187. The IC_{50} values varied between 1.1 µM to 20 µM for **45** and 2.1 µM to 25 µM for **46** without or together with increasing amounts of exogenous AA, respectively. In contrast, the respective isoquinoline-substituted derivatives **49** and **50** showed a different pattern. Especially compound **50** did not completely suppress 5-LO product formation up to 30 µM regardless of the substrate concentration.

4.3.2.2 Effects on 5-LO inhibition after stimulation by cell stress

The efficiency of 5-LO inhibitors, especially for non-redox type inhibitors was shown to depend on 5-LO activation in PMNL [200]. Chemical or osmotic cell-stress causes activation of 5-LO followed by formation of LTs and 5-HETE in PMNL. This mode of 5-LO activation was found to be independent from intracellular calcium concentration and is accompanied by strong activation of p38 MAPK [126]. Accordingly, it was investigated if the efficiency of the pyrazole derivatives was influenced when PMNL were stimulated by osmotic cell stress using a hyperosmotic solution of NaCl and 40 µM AA compared to 5-LO activation by A23187 (**Fig. 25**).

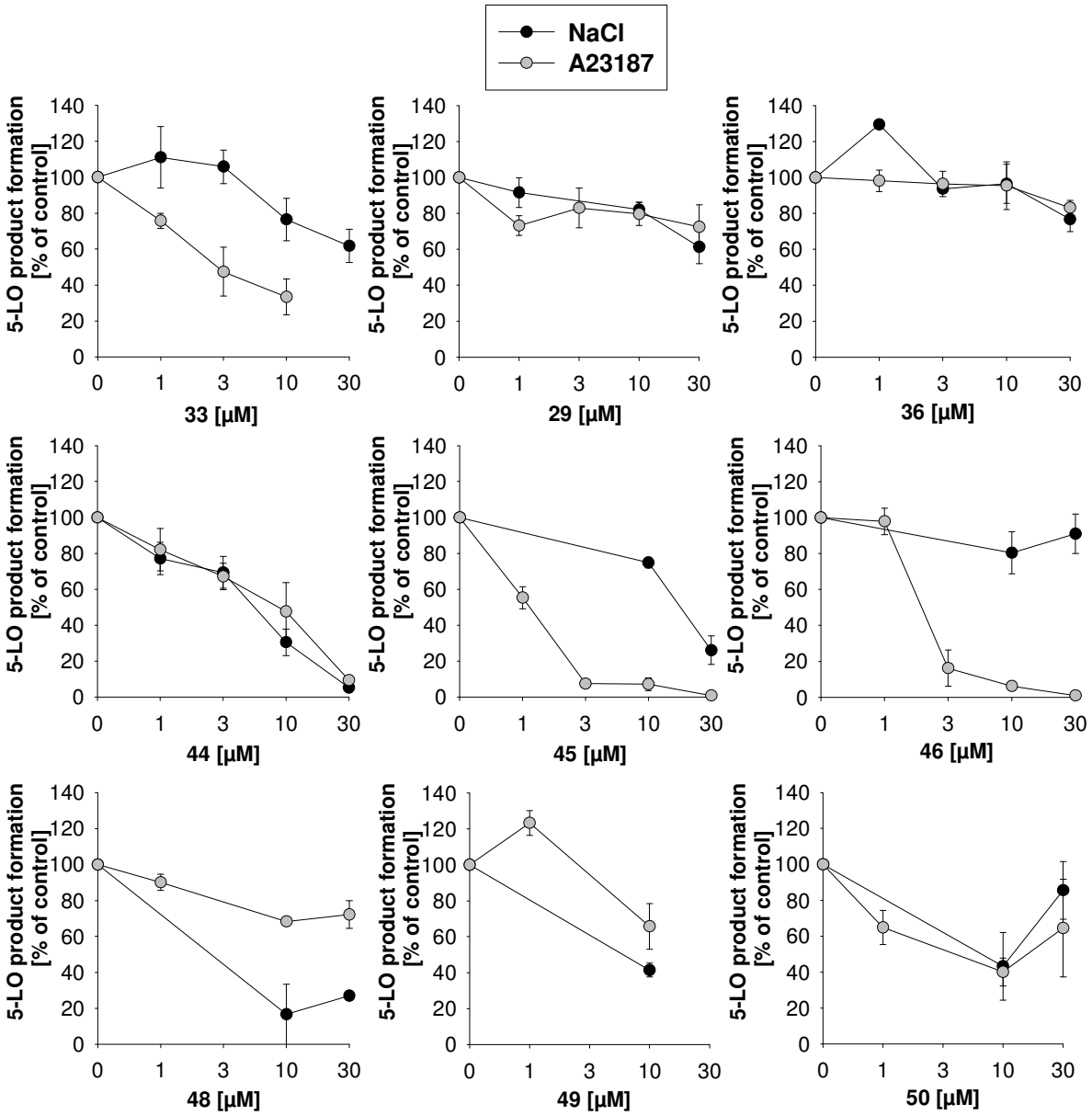


Fig. 25 Efficiency of the compounds after stimulation by osmotic cell stress vs. A23187
PMNL (5 x 10^6/ml) were pre-incubated for 15 min with the compounds or DMSO (max. 0.3%). Then, 0.3 M NaCl was added 3 min prior to 40 µM AA. After 10 min at 37 °C, 5-LO products were analysed by HPLC. Data shown as mean ± SEM; n =2-4

For **33** and **45**, an inhibitory effect of the compounds was observed after stimulation of PMNL by osmotic cell stress, but the potency was lowered more than 10-fold for both. The potency of the phenyl compound **44** and the isoquinoline derivative **49** did not differ between the different stimuli, suggesting that the inhibitory action is independently from the stimulus. No reduction of 5-LO product formation was observed for **46** after activation of 5-

LO by NaCl. Interestingly, the isoquinoline-substituted compound **48** was inactive in A23187-activated PMNL. However, an IC_{50} of approximately 3 µM was observed after addition of NaCl and 40 µM AA and 5-LO product formation was reduced to 25% at 30 µM. 5-LO products were neither diminished in PMNL after activation by A23187 nor by NaCl and 40 µM AA by **29** and **36** up to 30 µM. As mentioned before (see **4.3.2.1**), exogenous AA was required for these compounds to observe an inhibitory effect.

4.3.2.3 Influence of cellular components in the cell-free assay

As noted above, the compounds were more potent in the cell-based assay than in the cell-free assay. To assess the influence of factors remaining from the purification of recombinant 5-LO, the efficacy of the compounds in homogenates of E. coli expressing recombinant 5-LO (S40) and semi-purified 5-LO was compared (**Fig. 26**).

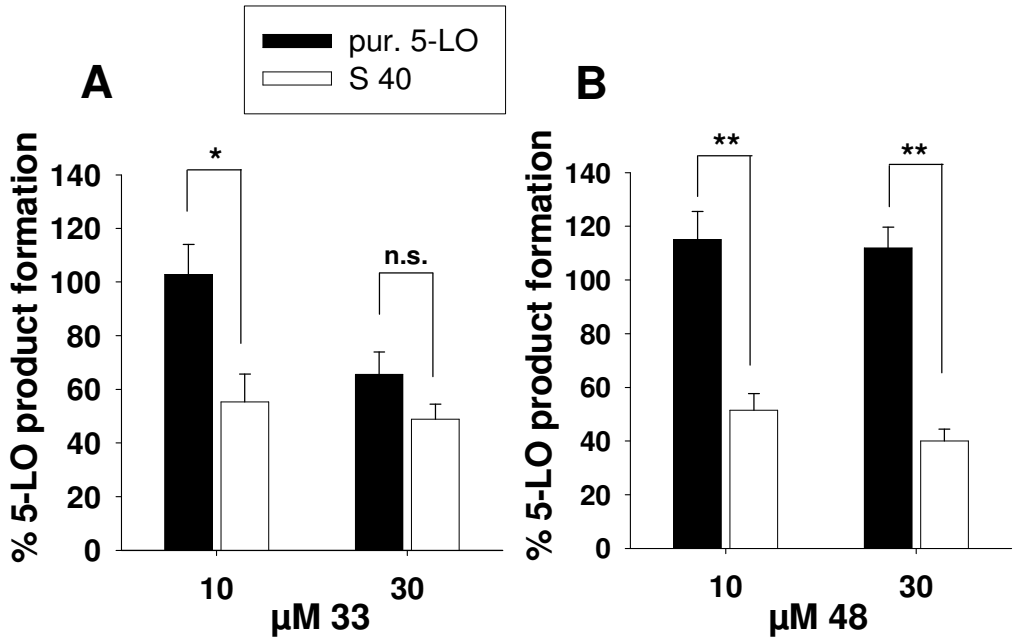


Fig. 26 Inhibition of purified 5-LO and S40 by compounds 33 (A) and 48 (B)
Aliquots of 40,000 x g homogenates of E. Coli expressing recombinant 5-LO (S40) or semi-purified 5-LO in PBS containing 1 mM EDTA and 1 mM ATP were pre-incubated for 10 min at 4 °C. Samples were pre-warmed for 30 sec at 37°C and 2 mM $CaCl_2$ and 20 µM AA were added to start the reaction. After 10 min at 37 °C, 5-LO products were analysed by HPLC. Data shown as mean ± SEM; n=3-7

*n.s.: not significant * $p < 0.05$, ** $p < 0.01$, unpaired t-test*

Recombinant 5-LO from E. coli homogenates (S40), but not semi-purified 5-LO was significantly inhibited by **33** at 10 and 30 µM versus control ($p < 0.05$). The absence of remaining cellular components in semi-purified 5-LO reduced the inhibitory effect of **33**, suggesting that these components somehow influence its potency (**Fig. 26A**). A similar effect, but with higher statistical significance was observed for the isoquinoline-substituted analogue **48** (**Fig. 26B**).

The corresponding phenyl derivative **44** showed an equal inhibition of 5-LO at 10 µM and 30 µM leading to remaining 5-LO activity of approximately 65%. However, this reduction was not considered as being statistically significant. **45** also inhibited 5-LO significantly compared to vehicle control in the cell-free assay, however only at high concentrations of 30 µM. The other pyrazole derivatives did not or only hardly inhibit 5-LO product formation in the cell-free assays up to 30 µM as described above (data not shown).

4.3.2.4 Influence of the redox tone on 5-LO inhibition in PMNL homogenates

Non-redox-type inhibitors of 5-LO, for example ZM 230487 or L-739,010, were shown to be very potent in intact cells, but lost their potency in cell-free systems and broken cell preparations. Exogenous addition of thiols such as GSH or DTT to reconstitute glutathione peroxidase restored the potency [139]. As shown above, the pyrazole derivatives inhibit 5-LO in intact PMNL, but show weak efficacy towards recombinant 5-LO. Therefore, it was investigated if the pyrazole compounds share properties with non-redox-type inhibitors. For this approach, homogenates of PMNL were prepared, 5 mM GSH was added and after pre-incubation with the compounds, 5-LO product formation was measured and compared to PMNL homogenates without addition of GSH (**Fig. 27**).

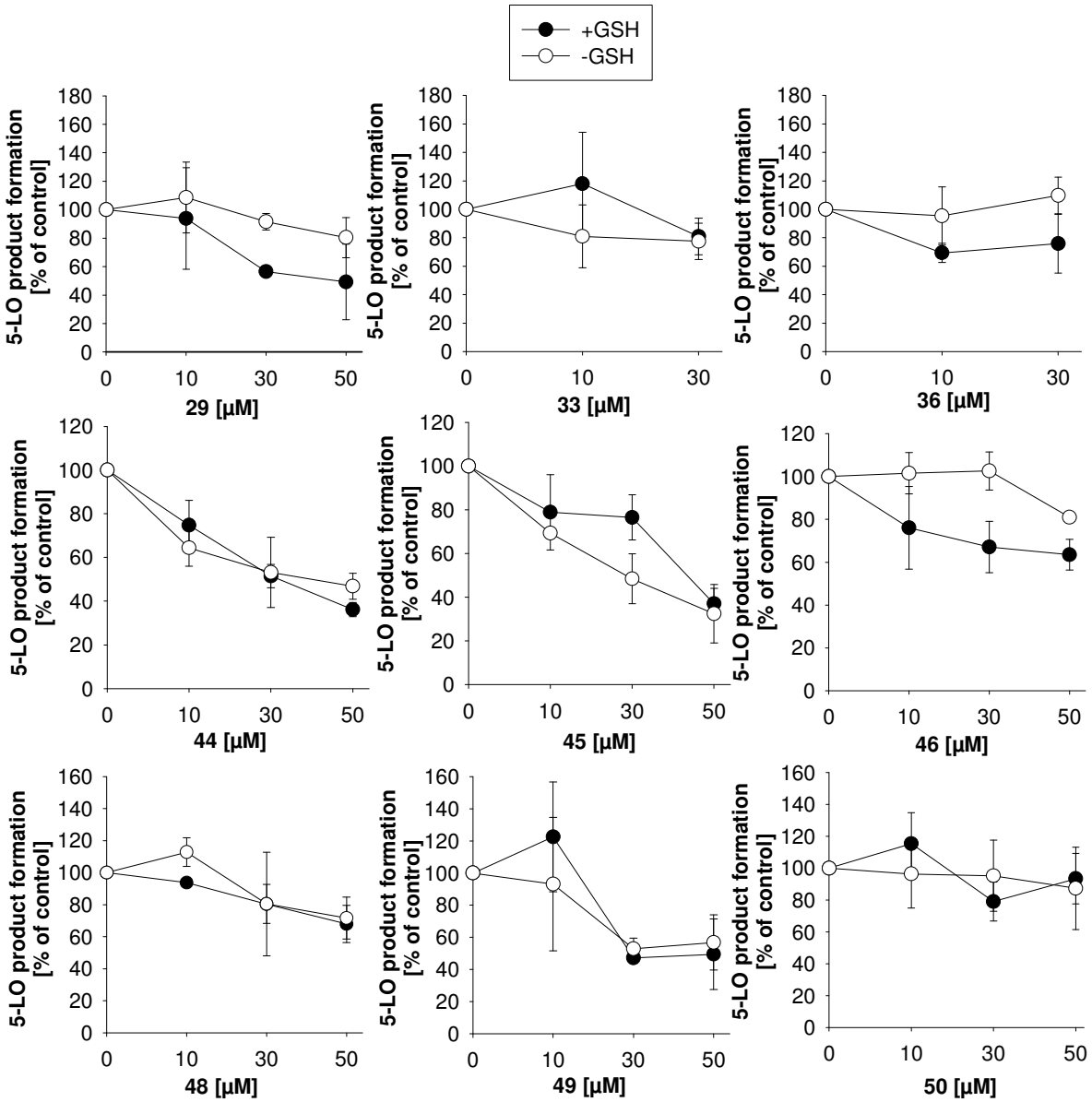


Fig. 27 Influence of GSH on 5-LO inhibition in PMNL homogenates
PMNL (5 x 10^6/ml) were resuspended in PBS containing 1 mM EDTA and disrupted. To aliquots (1 ml) of homogenates, 1 mM ATP and 5 mM GSH were added as indicated. After pre-incubation for 10 min at 4 °C with the compounds or DMSO (max. 0.3%), the reaction was started by addition of 40 µM AA. After 10 min at 37 °C, 5-LO products were analysed by HPLC. Data shown as mean ± SEM; n=2-4

For none of the compounds, a statistical significant inhibition of 5-LO in homogenates versus the DMSO control was found. In particular, for the phenyl-substituted derivatives **44-46**, neither a statistical significant inhibition versus control nor differences in the efficiencies with or without GSH was found. Alternatively, 1 mM DTT was added as thiol reagent to PMNL homogenates

pre-incubated with **44-46** leading to similar results (data not shown). These data suggest that the efficacy of the pyrazole derivatives is independent of the redox tone and the activity of GPx. Consequently, the selected pyrazol-propanoic acid analogs share no mechanistical properties of described non-redox-type inhibitors of 5-LO.

4.3.2.5 Inhibition of recombinant $cPLA_2$ and AA-release in monocytes

Endogenous substrate supply for 5-LO in the cell is regulated by $cPLA_2$. AA is cleaved by $cPLA_2$ from phospholipids in sn-2 position followed by conversion through 5-LO to the respective mediators. Inhibition at this early stage leads to reduced inflammatory effects due to reduced supply of 5-LO with AA [20]. Several compounds (**33**, **36**, **44-46**, **48-50**) were investigated at 10 µM in a cell-free assay with recombinant $cPLA_2$. Vesicles of arachidonate-containing lipids were incubated together with the test compounds and recombinant $cPLA_2$. Released AA was derivatized and quantified by HPLC.

All experiments with recombinant $cPLA_2$ and [^{3}H]-AA-labelled human monocytes described here were made by B. Jazzar in Tübingen and A. Schaible in Jena, respectively.

Except **44** and **48**, the compounds failed to inhibit recombinant $cPLA_2$ at 10 µM. As shown in **Fig. 28A**, **44** and **48** significantly inhibited recmbinant $cPLA_2$ in a concentration-dependent manner with IC_{50} values of 1.7 µM and 4.9 µM, respectively. 5 µM of pyrrolidine-1 was used as control inhibitor leading to 18.6 ± 3.2% remaining $cPLA_2$ activity. However, in a cell-based assay using [^{3}H]-labelled human monocytes, the release of AA by $cPLA_2$ was barely attenuated by 10 µM of **44** and **48**, whereas the control inhibitor pyrrolidine-1 inhibited AA-release at 1 µM, as expected [251] (**Fig. 28B**).

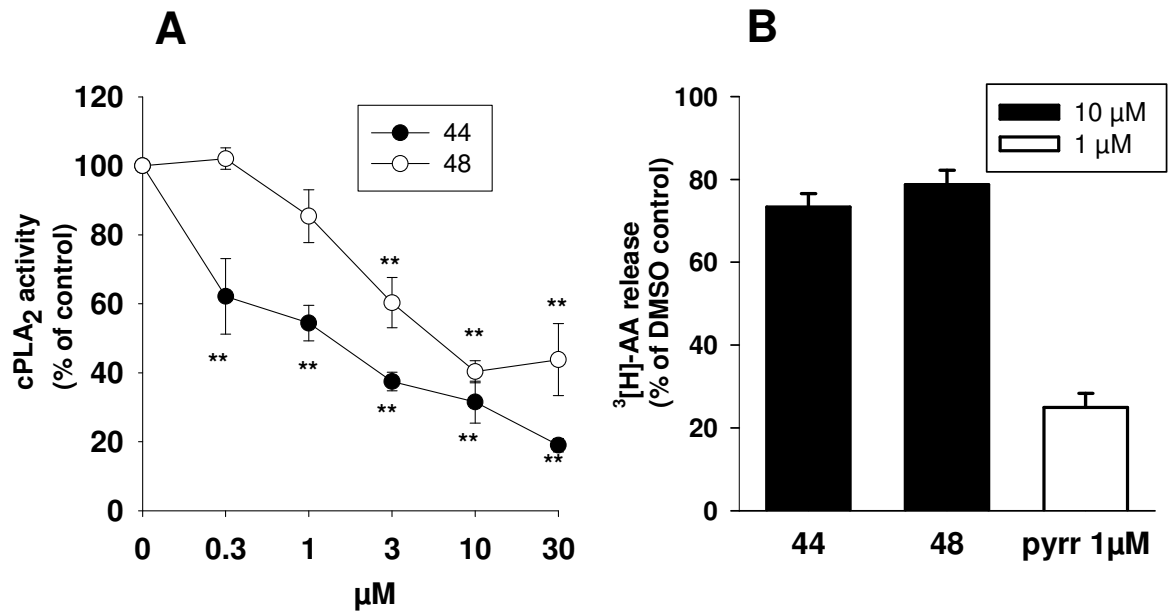


Fig. 28 Inhibition of recombinant cPLA$_2$ (A) and [^{3}H]-AA-release in monocytes (B)
Experiments were performed by B. Jazzar in Tübingen (A) and A. Schaible (B) in Jena, respectively. Recombinant human cPLA$_2$ was expressed in SF9 insect cells. LUVs of PAPC and POG (total lipid concentration 250 µM in 200 µl) were incubated with the test compounds and 1 mM $CaCl_2$. Reaction was started by addition of 500 ng recombinant cPLA$_2$. After 1 h at 37 °C, methanol was added, released AA was derivatized and analysed by HPLC (A) Alternatively, isolated human monocytes (2 x 10^6/ml) were incubated with 5 nM [^{3}H]-AA for 2 h at 37 °C and 6% CO_2. Cells were washed and resuspended in PGC buffer (1 mM $CaCl_2$), pre-incubated with the test compounds for 15 min at 37 °C and stimulated with 1 µM A23187 for 5 min at 37 °C. Released [^{3}H]-AA was measured in the supernatant on a scintillation counter (B); n=3-4 (A); n=2 (B); pyrr=pyrollidine-1

*** p < 0.01 vs. control; one-way-ANOVA with post-test*

4.3.2.6 Influence of Ca^{2+}depletion on the efficiency of the compounds

Ca^{2+} was shown to be an important factor to activate 5-LO. However, also in absence of intra- and extracellular calcium, 5-LO products were formed in PMNL after addition of AA. Moreover, stimulation of PMNL with exogenous AA alone leads to activation of p38 MAPK and ERK resulting in phosphorylation of the kinases [143]. In order to investigate if inhibition of 5-LO product formation depends on the presence of Ca^{2+}, intracellular $Ca^{2+}$was removed by addition of 30 µM BAPTA-AM and extracellular Ca^{2+} by 1 mM EDTA. Then, the cells were pre-incubated with the compounds following stimulation with 50 µM AA and 5-LO product formation was determined (**Fig. 29**).

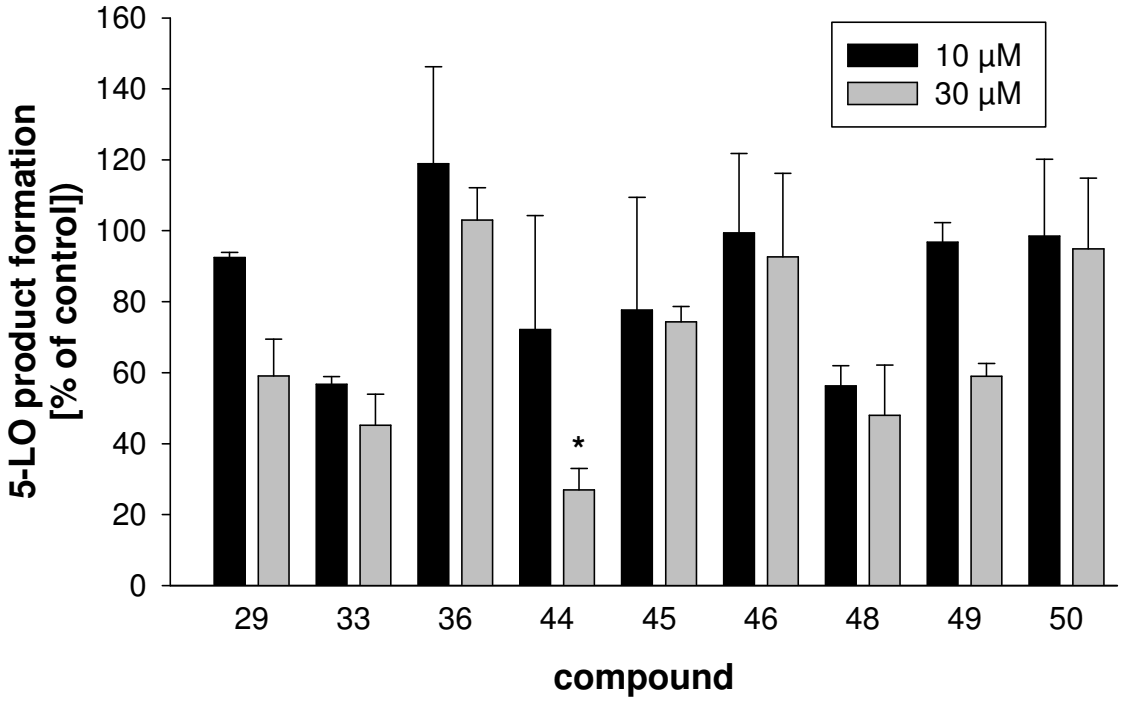


Fig. 29 5-LO inhibition in PMNL after depletion of intra-and extracellular Ca^{2+}
PMNL (5 x 10^6/ml) in PBS containing 1 mM EDTA (PBS-EDTA) were pre-incubated for 15 min at 37 °C with 30 µM BAPTA-AM. Then, test compounds or vehicle (DMSO max. 0.5%) were added for further 15 min at 37 °C and 50 µM AA was added. After 10 min at 37 °C, 5-LO products were analysed by HPLC. 5-LO product formation of DMSO control: 126.8 ± 11.1 ng/10^6 cells; Data shown as mean ± SEM; n=2;
** $p < 0.05$ vs. control; one-way-ANOVA with post-test*

No significant reduction of 5-LO product formation was observed for **36, 45, 46** and **50** up to 30 µM. In contrast, **33** and **48** reduced 5-LO product formation at 10 and 30 µM by half, suggesting that the inhibitory effect is independently from intra- and extracellular Ca^{2+}. Only at high concentrations of 30 µM, a reduction was seen for **29**, **44** and **49**. However, due to the low sample size, only a statistical significance for 5-LO inhibition of **44** at 30 µM was found.

4.3.2.7 Interference with 12-and 15-LO, COX and mPGES-1

The influence of the pyrazole compounds on other targets of the AA pathway such as 12-LO and 15-LO, mPGES-1, COX-1 and COX-2 was assessed in several cellular assays (**Table 18**). For 12- and 15-LO, formation of 12- and 15-H(P)ETE was measured in PMNL after stimulation with A23187 and 20 µM AA. The influence on COX-1 activity by the compounds was measured by 12-

HHT formation as a stable product of PGH_2 [234]. For the assessment of COX-2 activity, A549 lung carcinoma cells were stimulated with IL-1β to induce COX-2 expression [37]. After addition of AA, the amount of 6-keto-$PGF_{1\alpha}$ as COX-2 derived product was measured by EIA. mPGES-1 activity was determined by measurement of PGE_2 formation in microsomal preparations of IL-1β-stimulated A549 cells by HPLC.

Table 18 Effects of selected compounds on 12- LO, 15-LO, COX-1, COX-2 and mPGES-1
*Data shown as mean ± SEM of % remaining activity at 10 µM (unless otherwise stated) versus vehicle control (0.3% DMSO), n=3-8 * p<0.05, ** p<0.01 vs. control*
[a] 15- and 12-LO remaining activity at 10 µM, unless otherwise stated; 15-H(P)ETE was analyzed as product of 15-LO and 12-H(P)ETE as product of 12-LO in PMNL stimulated with 2.5 µM A23187+20 µM AA; n=2-3
[b] COX remaining activity at 10 µM, unless otherwise stated (% 12-HHT formation (COX-1) or 6-keto-$PGF_{1\alpha}$ formation (COX-2) of control); n=3-8
[c] mPGES-1 remaining activity at 10 µM (% PGE_2 of control); n=3-6

#	12-LO [a]	15-LO [a]	COX-1 [b]	COX-2 [b]	mPGES-1 [c]
29	90.4 ± 25.4	71 ± 45	102.2 ± 24.4	87.6 ± 13.2	103.0 ± 6.4
33	102.9 ± 13.4	175 ± 43	82.9 ± 3.2	84.2 ± 9.2	75.6 ± 7.0
36	77.1 ± 29.0	119.5 ± 7.5 (at 3 µM)	51.1 ± 3.9	106.7 ± 16.1	91.4 ± 4.6
44	118.4 ± 32.4	261 ± 49	21.0 ± 2.9 *	96.9 ± 11.7	83.8 ± 2.2
45	98.0 ± 6.1	196 ± 30	57.1 ± 12.1	86.3 ± 9.1	83.1 ± 2.9
46	67.2 ± 0.2	124 ± 22	10.6 ± 1.3 **	117.7 ± 19.5	105.6 ± 6.5
48	120.6 ± 21.7	168 (n=1)	8.2 ± 1.0	84.7 ± 19.6	75.0 ± 4.2
49	117.9 ± 16.3	167 ± 7.7	49.3 ± 6.8 (at 1µM)	88.4 ± 13.4	84.0 ± 4.1
50	82.7 ± 9.7	106.4 ± 2.5	16.7 ± 3.0 (at 1µM)	103.9 ± 7.1	88.0 ± 4.4

None of the compounds affected 12- or 15-LO remarkably up to 30 µM or mPGES-1 and COX-2 at 10 µM. COX-1-derived 12-HHT formation was not

reduced by the chloropyridazine-substituted derivatives **29** and **33** in isolated human platelets. Interestingly, **36** reduced 12-HHT formation to 51% at 10 µM. However, this inhibition was not considered statistically significant. In contrast, the phenyl- substituted compounds **44** and **46** potently reduced 12-HHT formation in platelets with IC_{50} values of 1.8 µM and 0.3 µM respectively (**Fig. 30A**).

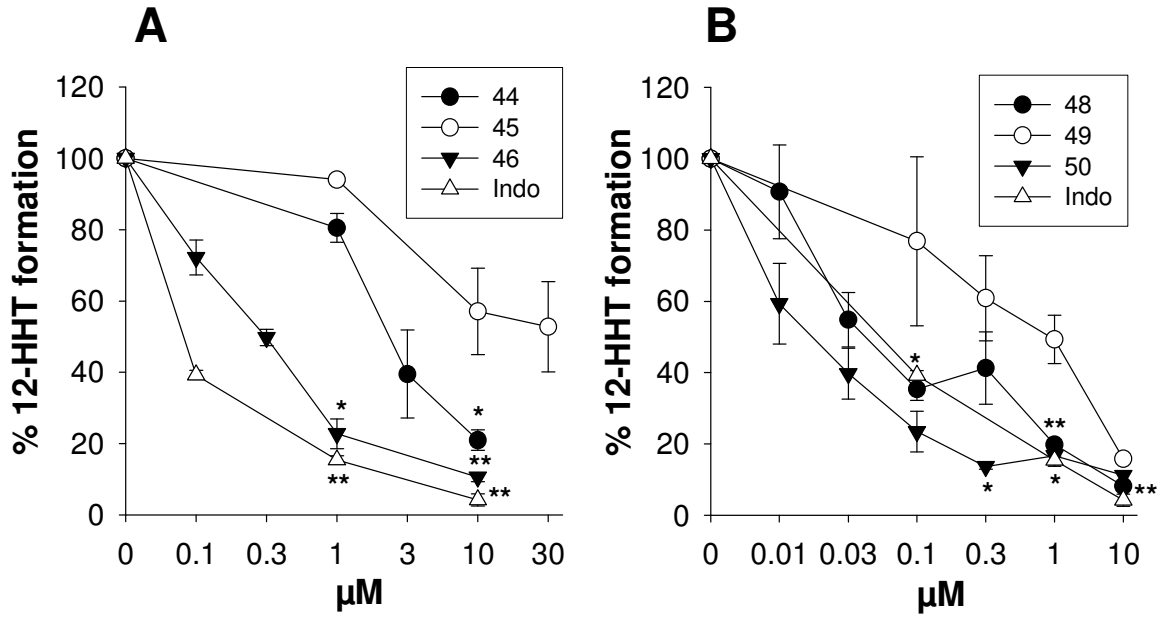


Fig. 30 Inhibition of COX-1 by phenyl-(A) and quinoline-substituted (B) pyrazole derivatives in human platelets

*Human platelets were pre-incubated for 4 min at RT and 1 min at 37 °C with compound or vehicle (DMSO 0.1%) and then stimulated with 5 µM AA. After 10 min at 37 °C, 12-HHT formation was analysed by HPLC. Indomethacin (Indo) was used as positive control. Data shown as mean ± SEM; n≥ 3 ; * p< 0.05, ** p< 0.01, one-way –ANOVA with post-test*

The isoquinoline-substitited derivatives **48** and **50** acted equally or even more potent on COX-1 with IC_{50} values of 0.043 µM and 0.014 µM than the control inhibitor indomethacine (IC_{50}= 0.047 µM) (**Fig. 30B**).

A dual inhibition of 5-LO and COX is supposed to have stronger anti-inflammatory effects and simultaneous less occurrence of gastrointestinal side effects [220]. Here, it seems apparent that a quinoline moiety at the pyrazole combined with a tert-butylbenzylpiperazine (**48**) or isopropylphenoxy substituent (**50**) favors a dual 5-LO/COX-1 inhibition.

4.3.2.8 Influence of the compounds on 5-LO translocation

An important step in 5-LO regulation is the subcellular localization of the enzyme. Upon activation by elevated Ca^{2+} concentration, 5-LO moves from the cytosolic compartment to the nuclear membrane where it colocalizes with $cPLA_2$. Then, AA is liberated from phospholipids in the nuclear membrane and transferred to FLAP [252]. It was recently shown that the distribution pattern of 5-LO is different in neutrophils from female and male donors [135]. The FLAP inhibitor MK-886 was the first compound described to block translocation of 5-LO [165]. Over time, several 5-LO inhibitors such as hyperforin [204], licofelone [238] or sulindac sulfide [253] have been described to reverse the agonist-induced translocation of 5-LO. Hence, the pyrazoles were investigated for possible effects on 5-LO translocation in female PMNL (**Fig. 31**).

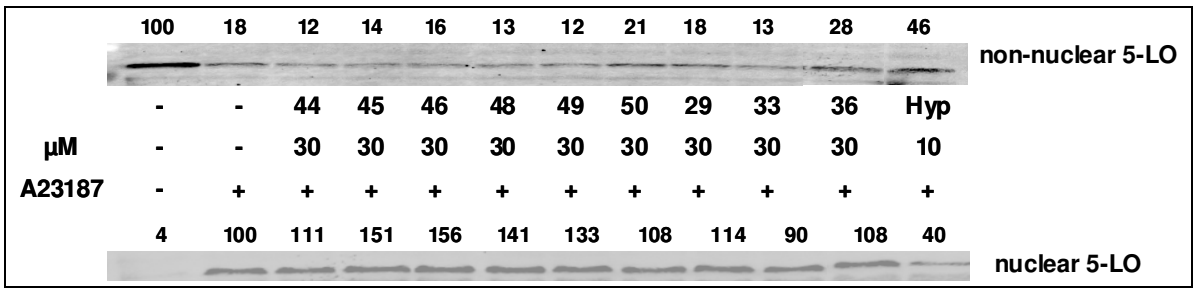


Fig. 31 Effects of pyrazole derivatives on 5-LO translocation
PMNL from female donors (3 x 10^7/ml) were pre-incubated for 15 min at 37 °C with vehicle (0.1% DMSO) or test compounds as indicated. After stimulation with 2.5 µM A23187 for further 10 min, reaction was stopped on ice and cells were lysed with buffer containing 0.1% NP-40 and fractionated by centrifugation. Soluble fractions (non-nuclear) and pellets (nuclear fraction) were separated by SDS-PAGE and analysed for 5-LO by Western blot. Hyperforin (Hyp) was used as positive control at 10 µM. Equal protein loading on the membranes was controlled by Ponceau staining (not shown).

Numbers above the panels represent arbitrary units of optical density compared to unstimulated sample (non-nuclear fraction) or stimulated sample (nuclear fraction) by densitometric analysis with ImageQuant® Software (GE Healthcare). The experiment shown is representative for at least two to three independent experiments.

The phenyl- and quinoline-substituted pyrazole derivatives **45**, **46**, **48** and **49** caused a slight increase of 5-LO in the nuclear fraction at 30 µM. For **45** and **46** this effect was already apparent at 10 µM (not shown). On the other hand, a slight reversal of translocation was apparent for **36** and **50**, however, at a very

high concentration of 30 µM. It has to be noted that the intensity of the reversal effect of the compounds on 5-LO translocation was not comparable to the observed effect of the control compound hyperforin at 10 µM.

4.3.2.9 Activation of ERK and p38 MAPK in neutrophils

Mitogen-activated protein kinases (MAPK) such as ERK2 and p38 MAPK contribute to the regulation of 5-LO [142],[140]. ERK2 and MK2, a p38 MAPK-regulated kinase, phosphorylate 5-LO at Ser663 and Ser271. This process is strongly upregulated *in vitro* after addition of polyunsatureted fatty acids [141]. In particular, the p38 MAPK pathway is related to inflammatory reactions [254]. Therefore, it was investigated if the pyrazole derivatives block 5-LO product formation by preventing phosphorylation of ERK or p38 MAPK after stimulation of the cells with the chemotactic peptide fMLP.

As shown in **Fig. 32A**, none of the compounds influenced the phosphorylation state of ERK2 up to a concentration of 30 µM. The MEK inhibitor U0126 was used at 3 µM as control and inhibited ERK-phosphorylation almost completely as expected [255]. In contrast, phosphorylation of p38 MAPK (p-p38) was enhanced approximately 5-fold by **44** at 30 µM compared to vehicle control as measured by densitometric analysis. Compound **48** also enhanced p38 MAPK phosphorylation at 30 µM, whereas the other compounds showed no effects up to 30 µM (**Fig. 32B**).

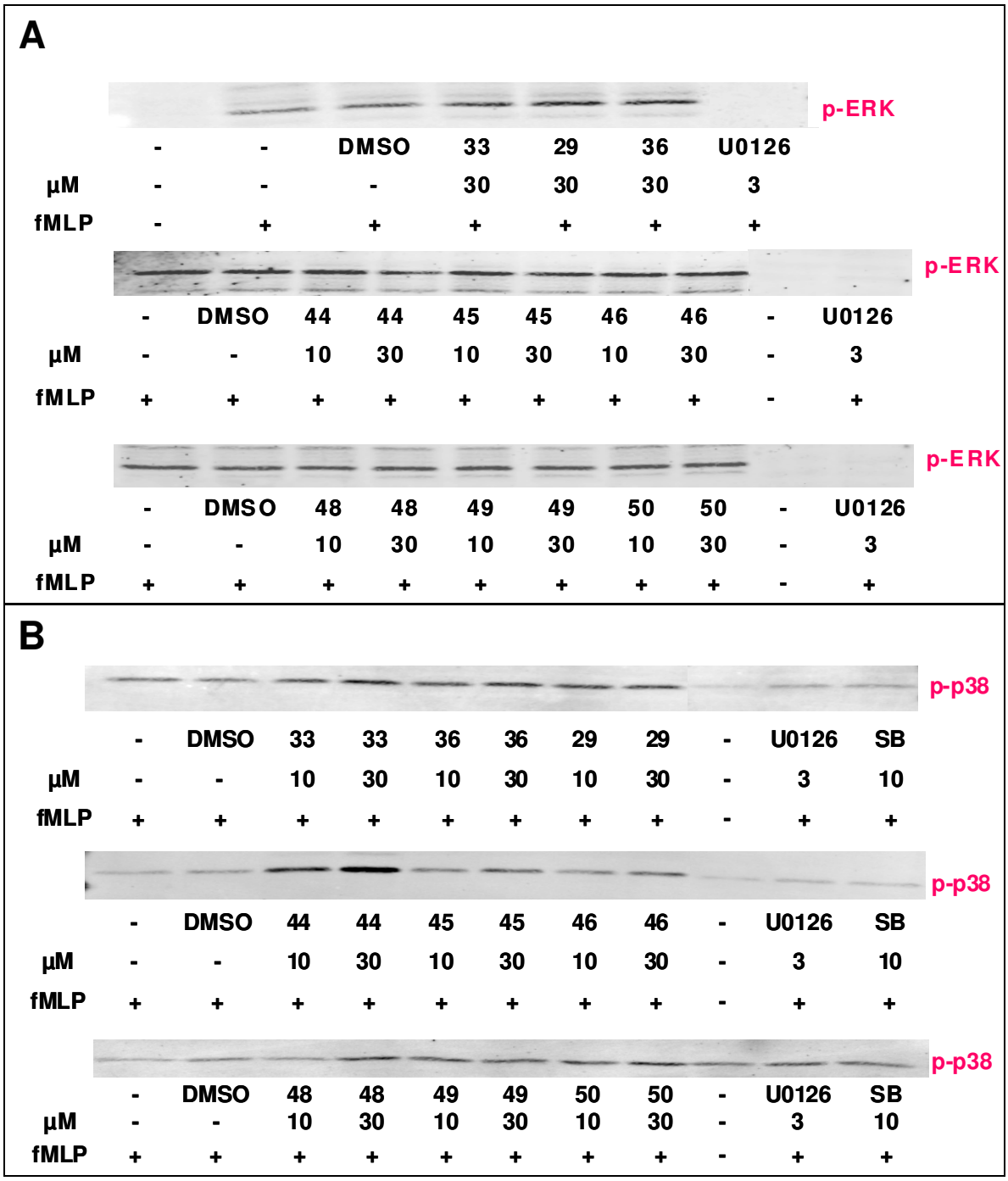


Fig. 32 Phosphorylation of ERK2 (A) and p38 MAPK (B) by pyrazole derivatives
PMNL (5 x 10^6 /100 µl PGC buffer) from female donors were pre-warmed at 37 °C for 3 min and pre-incubated with the test compounds or DMSO (max. 0.5%) for 15 min at 37 °C. Then, 1 µM fMLP was added and incubation was continued for 3 min. The reaction was stopped by addition of 100 µl of 2x SDS loading buffer. Proteins were detected with specific antibodies for phosphorylated ERK (p-ERK) and phosphorylated p38 MAPK (p-p38). Equal protein loading was checked by Ponceau staining (not shown). The results in the figure are representative for two to three independent experiments. SB=SB230580

The strong phosphorylation of p38 MAPK after addition of **44** might be caused by the compound itself and probably occurs also without fMLP stimulation. However, this aspect was not investigated.

4.3.2.10 Effects on 5-LO inhibition in human whole blood

Investigation of 5-LO product formation in a human whole blood assay may provide information regarding the *in vivo* action of a compound. Hence, human whole blood was stimulated with 30 µM A23187 after pre-incubation with the compounds and 5-LO products were measured (**Fig. 33**).

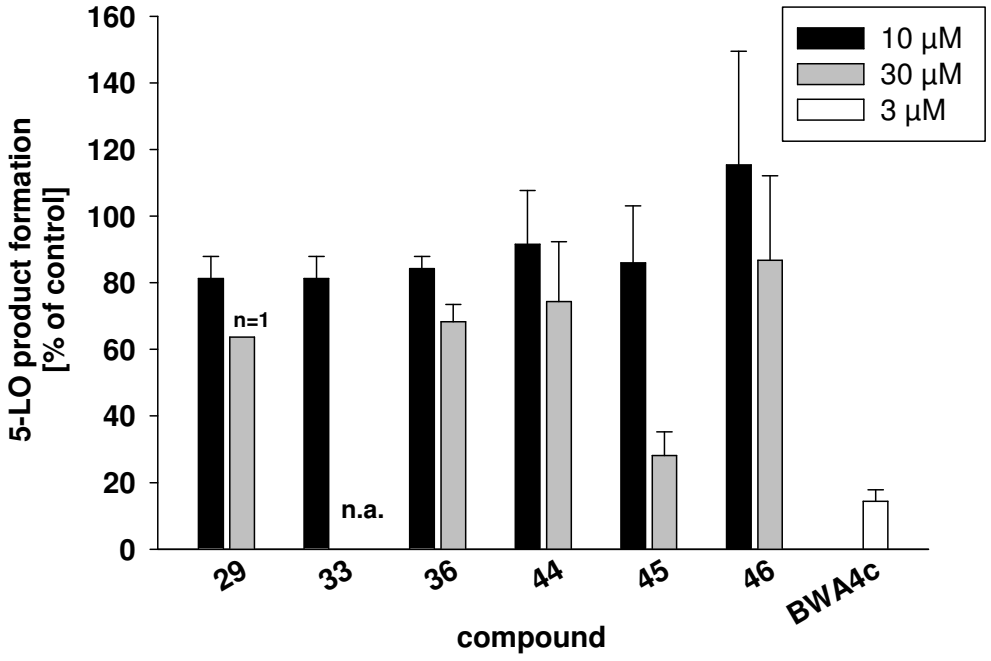


Fig. 33 5-LO inhibition in whole blood
Aliquots (1.5 ml) of human whole blood were pre-incubated with the compounds for 10 min at 37 °C and stimulated with 30 µM A23187. After 10 min at 37 °C, the reaction was stopped on ice and 5-LO products in the plasma were analysed by HPLC. Data shown as mean ± SEM; n=2-4, unless otherwise stated

5-LO product formation/ml plasma of DMSO control (100%): 265 ± 85 ng/ml; n.a.: data not available

At a concentration of 10 µM, 5-LO product formation was not significantly attenuated by any of the test compounds. However, **45** reduced 5-LO product formation at 30 µM to 28% of vehicle control. The control inhibitor BWA4C

clearly reduced 5-LO product formation already at 3 µM in the human whole blood assay as expected.

To sum up, derivatives of pyrazole-propanoic acid were found to be potent inhibitors of 5-LO product formation. They failed or only barely affect 5-LO in cell-free systems. In addition, depending on the substitution pattern of the pyrazole core, they reduced PG formation, showing properties of dual COX-1/5-LOX-inhibitors. However, the exact mode of action could not be elucidated, since their inhibitory properties cannot be clearly classified among the known 5-LO inhibitors.

4.4 Identification of LT biosynthesis inhibitors by virtual screening

Pharmacological intervention with LT biosynthesis to treat inflammatory, allergic and cardiovascular dieseases comprises several strategies. One approach involves the use of antagonists at the Cys-LT_1 receptor, e.g. montelukast, pranlukast or zafirlukast [81] or direct inhibition of 5-LO with zileuton as the only approved drugs [256]. Another approach aims at inhibition of 5-LO product biosynthesis with FLAP as promising target [152]. In order to identify new chemotypes for 5-LO/FLAP inhibitors, a virtual screening using a pharmacophore model that combines docking and ligand-receptor interaction fingerprints was applied.

4.4.1 Development of virtual screening and selection of test compounds

First, a set of 202 compounds known as FLAP inhibitors from literature was defined. This set was aligned to a complex of the inhibitor MK-591 bound to FLAP by using several modules of Molecular Operating Environment (MOE) software (MOE 2010.10, Chemical Computing Group, Canada). After alignment of the training set, a pharmacophore query was generated and used to screen libraries from Chemical Computing Group with 2.8 mio vendor compounds, whereof 1792 hits were identified. These hits were subjected for docking to the FLAP binding site. After refinement of the docking scores, 192 virtual hits were selected as candidates. The interactions of the compounds with the FLAP

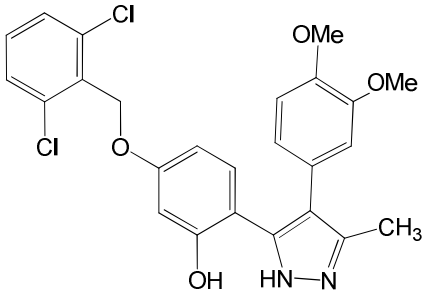
binding site were further characterized by using the protein-ligand interaction fingerprint (PLIF) application of MOE [257].

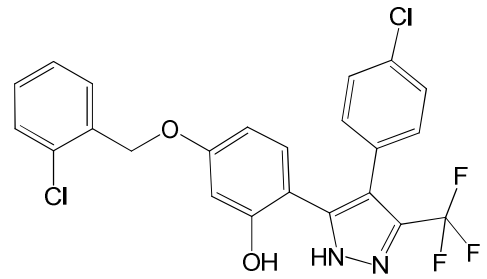
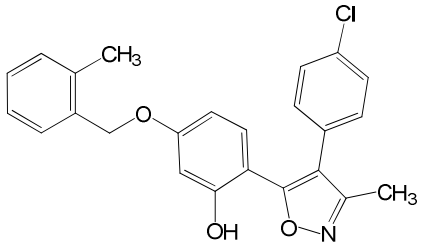
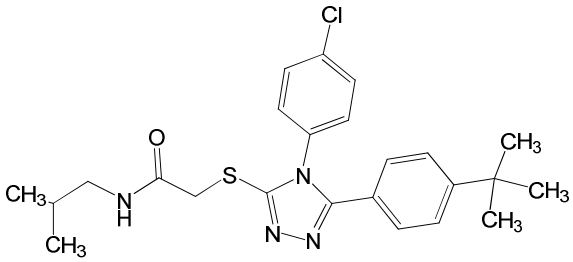
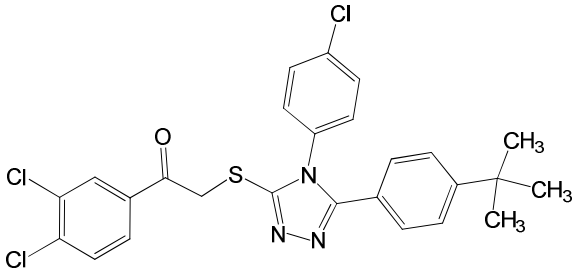
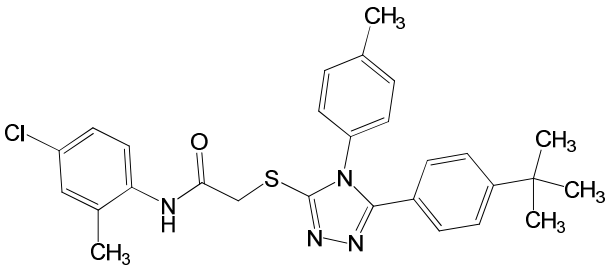
Development of the pharmacophore model, docking experiments and final selection of the test compounds were made by Dr. W. Altenhofen (Chemical Computing Group Cologne Germany) and the group of Prof. Banoglu (University of Ankara, Turkey), respectively. Finally, 8 compounds were selected based on chemical intuition and in consideration of the pharmacophore features for the evaluation of 5-LO inhibition .

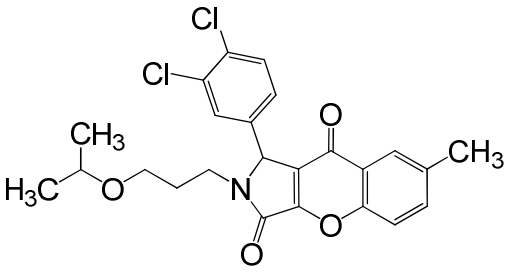
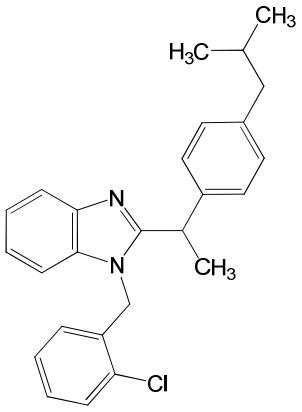
4.4.2 5-LO inhibition of virtual screening hits

The test compounds identified in the pharmacophore model were obtained from Ambinter Chemicals (Paris, France) and investigated for 5-LO inhibitory activity in intact human PMNL stimulated with A23187 and semi-purified 5-LO from E.coli homogenates expressing recombinant human 5-LO (**Table 19**).

Table 19 5-LO inhibition of selected screening hits
PMNL were stimulated with 2.5 µM A23187 or aliquots of E. coli homogenates expressing recombinant 5-LO (S40) were incubated with 2 mM $CaCl_2$ and 20 µM AA;
Data shown as % of remaining 5-LO activity at 10 µM (mean ± SEM) versus vehicle (0.3% DMSO); n ≥ 3

#	structure	PMNL % at 10 µM	S40 % at 10 µM
76		44 ± 6	58 ± 23

#	structure	PMNL % at 10 µM	S40 % at 10 µM
77		41 ± 10	53 ± 29
78		10 ± 8	16 ± 2
79		81 ± 7	62 ± 13
80		58 ± 13	116 ± 32
81		91 ± 30	85 ± 17

#	structure	PMNL % at 10 µM	S40 % at 10 µM
82		79 ±14	55 ± 14
83		4 ± 2	77 ± 15

Six compounds (**76-81**) share structural similarities. Their scaffolds consist of a central heterocyclic element such as pyrazole (**76,77**), isoxazole (**78**) or triazole (**79-81**) substituted with diaryl residues such as dimethoyxyphenyl (**76**) or chlorophenyl (**77-81**). In contrast, the scaffold of **82** contains a dihydro-chromeno-pyrrol-dione with an aliphatic 3-isopropoxypropyl and an aromatic 3,4-dichlorophenyl substituent. The chlorobenzyl-substituted benzimidazole scaffold of **83** contains shows structural similarity to the indole-based FLAP inhibitor MK-886.

Compounds **76, 77** and **82** seem to act as direct, but weak inhibitors of 5-LO with IC_{50} values of ≥ 10 µM in the cell-free assay. The 2-(4H-1,2,4-triazole-3-ylthio)acetic acid derivatives **79**-**81** inhibited 5-LO product formation weakly with IC_{50} values >10 µM in the cell-based as well as in the cell-free assay. In contrast, the diaryl-substituted methyl-isoxazole **78** reduced 5-LO product formation potently in both assays. Additionally, the benzimidazole derivative **83**

almost completely blocked 5-LO product formation in PMNL at 10 µM. However, in the cell-free assay, **83** recombinant 5-LO was barely affected at 10 µM. The results of further experiments with these compounds will be presented in detail in the following chapter.

4.4.3 Pharmacological characterization of identified screening hits

4.4.3.1 Effects of compound 78 on 5-LO product formation

The diarylmethylisoxazole derivative **78** ((2-(4-(4-chlorophenyl)-3-methyl-isoxazol-5-yl)-5-(2-methyl-benzyloxy)phenol) was identified as a potent inhibitor of 5-LO and subjected to further investigations. In the cell-based assay, 5-LO product formation was concentration-dependently reduced by **78** (IC_{50} = 4.4 µM). Addition of 40 µM exogenous AA together with 2.5 µM A23187 attenuated the inhibitory effect in PMNL leading to an IC_{50} of about 6 µM. Recombinant semi-purified 5-LO was also inhibited by **78** leading to around 40% of remaining activity up to 30 µM (**Fig. 34**).

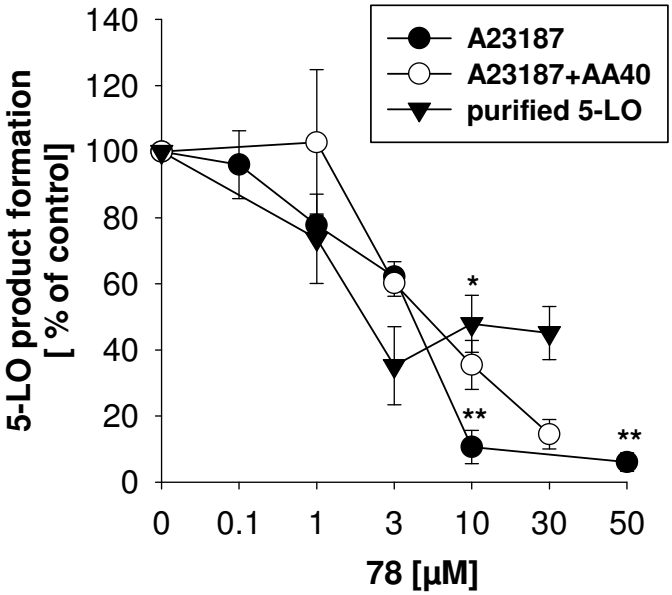


Fig. 34 5-LO Inhibition of compound 78 in PMNL and cell-free assay
*PMNL (5 x 10^6/ml with exogenous AA or 1 x 10^7/ml without exogenous AA) in PGC buffer were pre-incubated with compound 78 or DMSO for 15 min at 37 °C. Then, 2.5 µM A23187 was added with or without 40 µM AA as indicated. Alternatively, recombinant semi-purified 5-LO was pre-incubated for 10 min at 4 °C. Samples were pre-warmed for 30 sec at 37 °C and 2 mM $CaCl_2$ and 20 µM AA were added to start the reaction. After 10 min at 37 °C, 5-LO products were analysed by HPLC. Data shown as mean ± SEM; n= 2-7 * p< 0.05, ** p< 0.01, one-way –ANOVA with post-test*

4.4.3.2 Effect of compound 78 on COX-1 and 12-LO product formation

The influence of **78** on COX-1-derived prostaglandin formation was evaluated by the measurement of 12-HHT in isolated human platelets. **78** caused no reduction of 12-HHT formation at 10 µM (97 ± 9% remaining 12-HHT formation compared to DMSO control). 12-HETE formation, resulting from oxygenation of AA by 12/15-LO in neutrophils or p12-LO in adhering platelets, was barely attenuated by 10 µM of **78** (72% remaining 12-HETE formation compared to DMSO control).

4.4.3.3 Effects of compound 83 on 5-LO product formation

As second active compound, **83** (1-(2-chlorobenzyl)-2-(1-(4-isobutylphenyl) ethyl)-1H-benzo[d]imidazole) was identified in the virtual pharmacophore model. In A23187-stimulated PMNL, 5-LO product formation was reduced by

83 in a concentration-dependent manner with an IC_{50} of 0.31 µM. However, after addition of 20 or 40 µM of exogenous AA together with A23187, 5-LO activity was not completely suppressed by **83** up to 30 µM (**Fig. 35A**). In preparations using crude homogenates (S40) or semi-purified recombinant 5-LO, only a weak inhibition of 5-LO up to 50 µM was apparent, excluding a direct interaction of **83** with 5-LO (**Fig. 35B**).

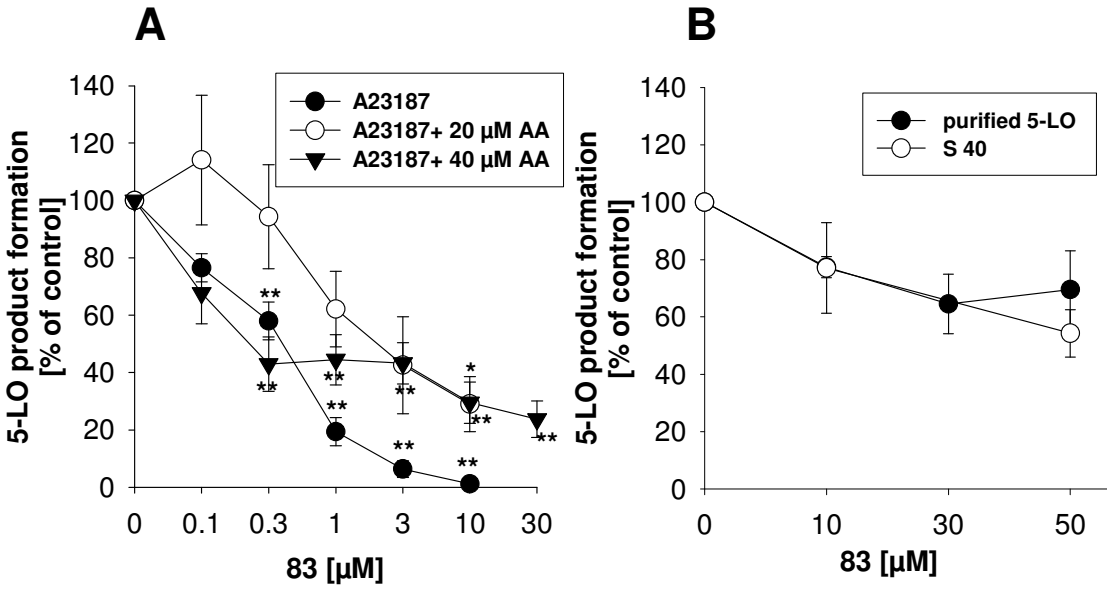


Fig. 35 Effect of 83 on 5-LO product formation in PMNL (A) and recombinant 5-LO (B)
*PMNL (5 x 10^6/ml with exogenous AA) or 1 x 10^7/ml without exogenous AA) were pre-incubated with 83 or DMSO for 15 min at 37 °C. Then, 2.5 µM A23187 was added without or together with AA. (A) Alternatively, aliquots (1 ml) of supernatants of E.coli homogenates expressing recombinant 5-LO (S40) or semi-purified 5-LO were diluted in 1 ml PBS, pH 7.4 and 1 mM EDTA and pre-incubated with 83 or DMSO for 10 min at 4 °C. Samples were pre-warmed for 30 sec at 37 °C and 2 mM $CaCl_2$ and 20 µM AA were added to start the reaction (B). After 10 min at 37 °C, 5-LO products were analysed by HPLC. Data shown as mean ± SEM; n ≥ 3; * p< 0.05, ** p< 0.01 vs. DMSO control, one-way –ANOVA with post-test*

High concentrations of AA *in vitro* attenuate the influence of FLAP in the cell and thus, AA is directly converted by 5-LO. Hence, 5-LO product formation is not further reduced at high inhibitor concentrations and remains on a level around 20 to 30%. This fact was observed for **83** and has been described for licofelone and MK-886 [238]. In addition, FLAP inhibitors do not inhibit 5-LO in cell homogenates or recombinant 5-LO. Accordingly, **83** failed to reduce 5-LO product formation in homogenates of PMNL up to 50 µM. Addition of 5

mM GSH to the cell homogenates had no influence on the potency, excluding that **83** acts as a non-redox-type inhibitor of 5-LO (data not shown).

4.4.3.4 Effects of compound 83 on 12- LO, 15-LO and PG formation

12-/15-LO, also referred to as 15-LO-1, oxygenates AA at position 12 and 15 to 12- and 15- H(P)ETE and is expressed, for example, in eosinophils [258]. Furthermore, platelet-type-12-LO (p-12-LO), forming 12-HETE is present from adherent platelets in the cell preparation. Thus, the effect of **83** on 12- and 15-HETE formation was investigated. No inhibitory effect neither on 12- nor on 15-HETE formation by **83** was observed up to 30 µM (**Fig. 36**). Interestingly, 15-HETE formation was even increased at higher concentrations of **83**, caused by a shunt of AA and thus conversion by 15-LO.

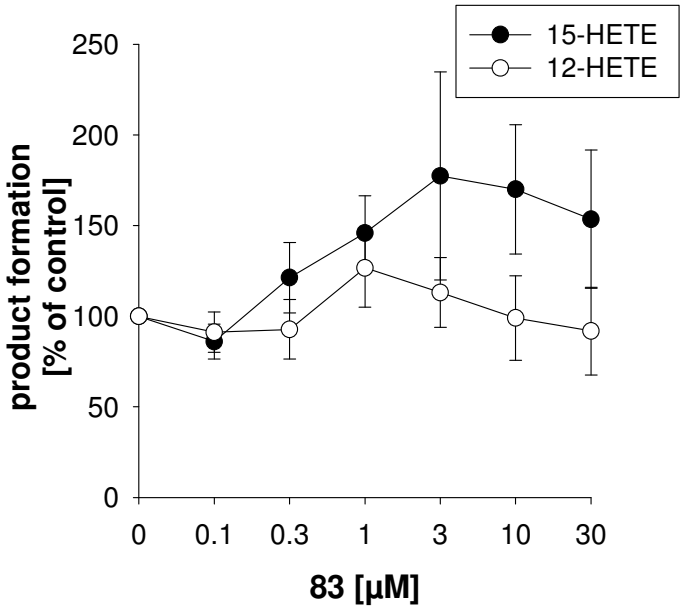


Fig. 36 Effects of 83 on 12-LO and 15-LO product formation
12- and 15-HETE formation were measured in PMNL stimulated with 2.5 µM A23187 + 40 µM AA and were analysed by HPLC. Data shown as mean ± SEM; n ≥ 3

Next, the effect of **83** on PG formation was investigated. For this purpose, formation of 12-HHT as non-enzymatic COX-1 derived product of PGH_2 was measured. For determination of COX-2 inhibition, 6-keto-$PGF_{1\alpha}$ as stable product of PGI_2 was measured by EIA. Neither COX-1 nor COX-2-derived

product formation was affected by **83** up to 50 µM. Additionally, mPGES-1-derived PGE_2 formation was not altered by **83** at 10 µM (data not shown).

4.4.3.5 Influence of compound 83 on 5-LO translocation

5-LO translocation describes the movement of the enzyme in the cell from the cytosolic compartment to the nuclear membrane after activation by calcium or other stimuli. FLAP colocalizes with 5-LO and $cPLA_2$ at the nuclear membrane, and converts AA to LTs and 5-HETE [161]. FLAP inhibitors such as MK-886 have been reported to inhibit agonist-induced subcellular translocation of 5-LO from the cytosol to the nuclear membrane [165]. Due to a certain structural similarity of **83** with MK-886, the influence on cellular 5-LO redistribution was investigated. As shown in **Fig. 37**, the translocation of 5-LO in PMNL to the nuclear membrane was partly reversed by **83** starting from 1 µM.

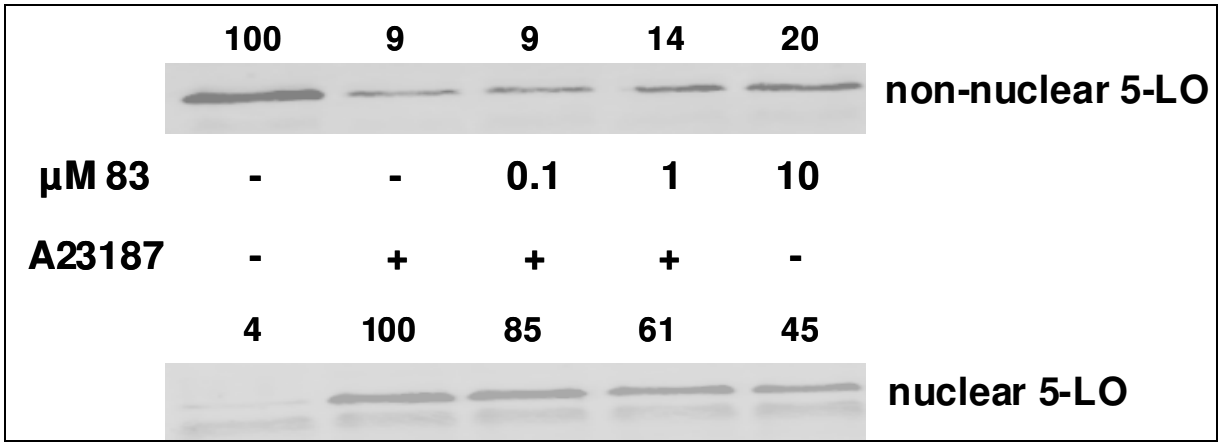


Fig. 37 Influence of compound 83 on 5-LO translocation
PMNL from female donors (3x 10^7/ml) were pre-incubated for 15 min at 37 °C with vehicle (0.1% DMSO) or 83 as indicated. After stimulation with 2.5 µM A23187 for further 10 min, the reaction was stopped on ice and cells were lysed with buffer containing 0.1% NP-40 and fractionated by centrifugation. Soluble fractions (non-nuclear) and pellets (nuclear fraction) were separated by SDS-PAGE and analysed for 5-LO by Western blot. Equal protein loading on the membranes was controlled by Ponceau staining (not shown). Numbers above the panels represent arbitrary units of optical density compared to unstimulated sample (non-nuclear fraction) and stimulated sample (nuclear fraction) by densitometric analysis with EttanDIGE® system and ImageQuant® Software (GE Healthcare). The experiment shown is representative for at least 3 independent experiments.

4.4.3.6 5-LO inhibition of compound 83 in human whole blood

In order to study the inhibitory action under more physiological conditions, **83** was investigated in the human whole blood assay. Here, the formation of LTB_4 and 5-HETE in the plasma was reduced by **83** in a concentration-dependent manner with an IC_{50} of 10.1 µM (**Fig. 38**). Albeit the potency of **83** in whole blood is 40-fold lower compared to human neutrophils, this concentration might be reached in the blood plasma after oral administration of the compound.

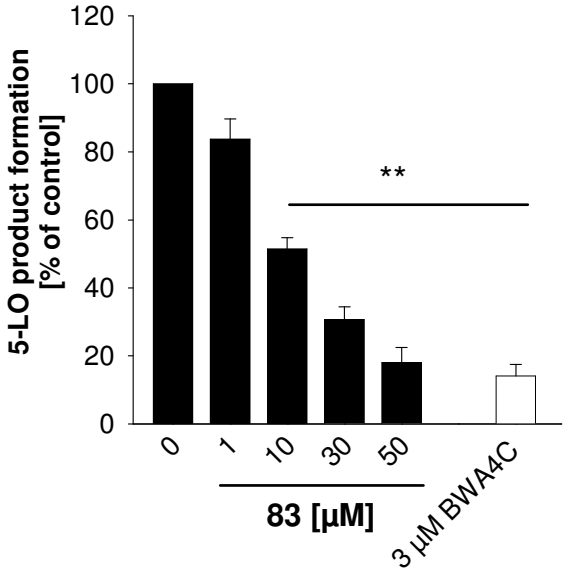


Fig. 38 Effects of 83 on 5-LO inhibition in human whole blood
*Aliquots of human whole blood (1.5 ml) were pre-incubated with 83 or vehicle (DMSO 0.1%) for 10 min at 37 °C and then stimulated with 30 µM A23187. After 10 min at 37 °C, the reaction was stopped on ice and 5-LO products in the plasma were analysed by HPLC. Data shown as mean ± SEM; n = 3-5. ** p< 0.01 vs. DMSO control, one-way –ANOVA with post-test*

4.4.3.7 *In vivo* experiments

An *in vivo* characterization of **83** was carried out in the carrageenan-induced rat pleurisy, an animal model of acute inflammation in the group of Prof. L. Sautebin at the University of Naples. As shown in **Table 20,** 10 mg/kg of **83** reduced significantly the amount of exudate volume, the number of inflammatory cells and LTB_4 formation in the exudate compared to vehicle-treated rats. Admittedly, in a comparable experiment, 10 mg/kg zileuton showed

a stronger anti-inflammatory effect in this *in vivo* model than **83** (for data of the control inhibitor zileuton see **Table 7**.

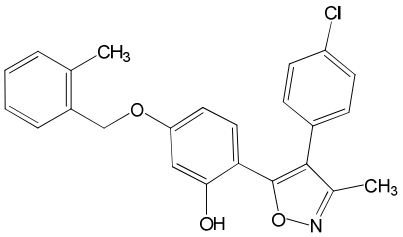
Table 20 In vivo effects of compound 83 on carrageenan-induced pleurisy in rats
30 min before intrapleural injection of carrageenan, male rats (n=10 for each experimental group) were treated i.p. with 10 mg/kg 83 or vehicle (DMSO 4%). Exudate volume, LTB_4 and inflammatory cell accumulation in pleural cavity were assessed 4 h after carrageenan injection. Data are expressed as mean ± SEM, n = 10
***p<0.01; ***p<0.001 vs. vehicle (t student)*
Animal experiments were made in the group Prof. L. Sautebin, University of Naples, Italy

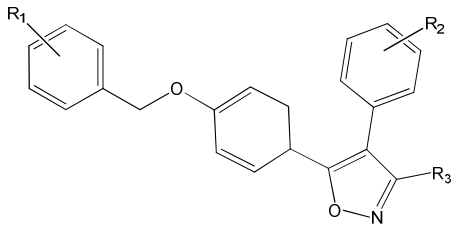
treatment	**exudate volume (ml)**	**inflammatory cells ($\times 10^6$)**	**LTB_4 (ng/rat)**
vehicle	0.325 ± 0.021	50.4 ± 2.67	0.512 ± 0.04
83 (10 mg/kg)	0.217 ± 0.026 ** (33 %)	36.8 ± 2.10 *** (27 %)	0.366 ± 0.02 ** (29 %)

4.4.4 SAR of 4,5-diarylisoxazole derivatives

Next, **78** was chosen as lead compound for structural modifications and subjected to SAR studies. Modifications of **78** involved the removal of the phenolic hydroxyl group and repositioning of the methyl group at the benzyloxy moiety to para-position (**84**) or replacement by fluorine at C-3 (**86, 88, 89, 92**). Furtheron, the chlorine at the phenyl ring connected with the isoxazole was varied between para- (**78, 85, 86, 89, 90**) and meta-position (**87, 88, 91, 92**) (**Table 21**).

Table 21 5-LO inhibition of 4,5-diarylisoxazole derivatives
PMNL were stimulated with 2.5 µM A23187 or aliquots of semi-purified recombinant 5-LO (purified 5-LO) were incubated with 2 mM $CaCl_2$ and 20 µM AA; Data shown as % of remaining 5-LO activity at 10 µM (mean ± SEM) versus vehicle (0.3% DMSO) or IC_{50}
n ≥ 3; n.i: no inhibition at 10 µM

#	structure	5-LO activity (% of control at 10 µM)	IC_{50} [µM] (PMNL)	IC_{50} [µM] pur. 5-LO)
78		3.0 ± 0.7	4.4	2.6



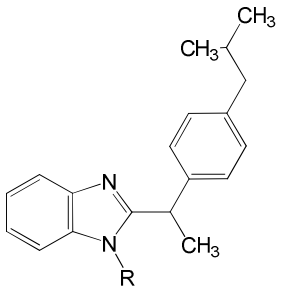
#	R1	R2	R3	5-LO activity (% of control at 10 µM)	IC_{50} [µM] (PMNL)	IC_{50} [µM] (pur. 5-LO)
84	4-CH_3	4-Cl	COOEt	61.4 ± 12.4	> 10	n.i.
85	2-CH_3	4-Cl	COOEt	71.9 ± 10.3	> 10	n.i.
86	3-F	4-Cl	COOEt	55.3 ± 12.1	> 10	n.i.
87	2-CH_3	3-Cl	COOEt	83.4 ± 16.2	> 10	n.i.
88	3-F	3-Cl	COOEt	80.8 ± 11.8	> 10	n.i.
89	3-F	4-Cl	COOH	19.6 ± 9.5	1.2	0.58
90	2-CH_3	4-Cl	COOH	13.1 ± 3.9	1.7	0.7
91	2-CH_3	3-Cl	COOH	0.7 ± 0.7	2.0	1.15
92	3-F	3-Cl	COOH	8.4 ± 5.1	1.6	0.75

Interestingly, the introduction of ethyl carboxylate instead of a methyl group at C-3 of the isoxazole (**84-88**) led to a complete inactivity of the compounds in intact cells and in the cell-free assay. In contrast, the respective free carboxylic acids (**89-92**) inhibited 5-LO product formation potently with IC_{50} values between 1.2 and 2 µM in PMNL. For example, meta-positioning of flourine enhanced the potency of **89** almost 4-fold in PMNL compared to the lead compound **78**. Interestingly, the IC_{50} values for **89-92** were approximately 2-fold higher in the cell-based assay compared to semi-purified 5-LO. To sum up, the results propose the 4,5-diarylisoxazole carboxylic acid analogs as direct 5-LO inhibitors. Since 5-LO product formation from recombinant 5-LO as well as in PMNL was affected, the 4,5-diarylisoxazoles still may share characteristics with FLAP inhibitors but also seem to directly interfere with 5-LO.

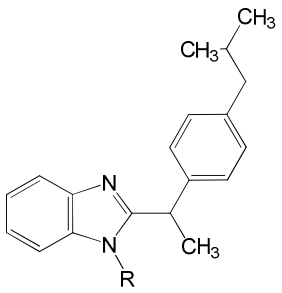
4.4.5 SAR of benzimidazole derivatives

In order to study the influence of structural modifications and define SARs, **83** was defined as lead compound and a series of derivatives was synthesized by the group of Prof. E. Banoglu, University of Ankara, Turkey. **Table 22** shows the influence on 5-LO inhibition after modifications at N-1 of the benzo[d] imidazole scaffold in A23187-activated PMNL. Removal of the 2-chlorine at the N-benzyl of the lead compound **83**, yielding an unsubstituted ring (**93)** decreased the potency to 0.98 µM. Repositioning of the chlorine to the para-position (**94)** caused approximately 15-fold loss of activity compared to the lead compound. Other residues at C-4 of the benzyl ring were tolerated (**95-101**), but failed to increase the potency on 5-LO with IC_{50} values between 2.3 and 6.0 µM. Especially bulky substituents such as isopropyl (**102**), tert-butyl (**103**) or an acetoxygroup (**104**) further diminished the potency resulting in IC_{50} values from 7.2 µM to greater than 10 µM.

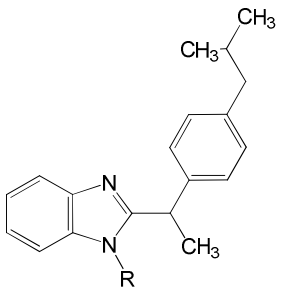
Table 22 5-LO inhibition of benzoimidazole derivatives based on lead compound 83
PMNL were stimulated with 2.5 µM A23187; Data shown as % of remaining 5-LO activity at 1 µM or 10 µM (mean ± SEM) versus vehicle (0.3% DMSO), unless otherwise stated; n ≥ 3
n.i.: no inhibition up to 10 µM



#	R	remaining 5-LO activity (% ± SEM at 1 µM)	IC_{50} [µM] or remaining 5-LO activity (% ± SEM at 10 µM)
83	2-chlorobenzyl	13.2 ± 4.4	0.31
93	benzyl	47.7 ± 6.2	0.98
94	4-chlorobenzyl	66.5 ± 11.7	4.8
95	4-methoxybenzyl	63.0 ± 7.8	3.8
96	4-methylbenzyl	67.1 ± 8.9	2.9
97	4-cyanobenzyl	100.4 ± 35.2	2.3
98	4-iodobenzyl	78.2 ± 3.6	5.0
99	4-bromobenzyl	79.7 ± 6.4	6.0
100	4-fluorobenzyl	65.8 ± 10.7	3.4
101	4-(triflouromethyl)benzyl	83.9 ± 5.6	4.9
102	4-isopropylbenzyl	83.8 ± 6.6	7.2
103	4-tert-butylbenzyl	98.3 ± 7.0	n.i. (77.8 ± 10.6)



#	R	remaining 5-LO activity (% ± SEM at 1 µM)	IC_{50} [µM] or remaining 5-LO activity (% ± SEM at 10 µM)
104	4-acetoxybenzyl	99.8 ± 13.1	>10 (56.5 ± 24.1)
105	3-acetoxybenzyl	109.2 ± 10.8	n.i. (86.7 ± 22.1)
106	3-fluorobenzyl	71.1 ± 11.9	4.0
107	3-cyanobenzyl	91.1 ± 18.5	> 10 (60.8 ± 31.4)
108	2-hydroxybenzyl	84.8 ± 8.3	4.5
109	2-methoxybenzyl	9.2 ± 4.9	0.44
110	2-acetoxybenzyl	8.4 ± 3.1	0.25
111	2-cyanobenzyl	43.5 ± 9.8	0.45
112	2-methylbenzyl	18.1 ± 10.0	0.5
113	2-fluorobenzyl	43.9 ± 7.3	0.7
114	2,6-dichlorobenzyl	89.4 ± 2.2	4.4
115	2,4-dichlorobenzyl	24.6 ± 12.5	0.5
116	3,4,5-trimethoxybenzyl	120.3 ± 4.7	9.4



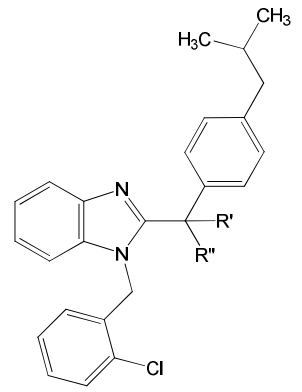
#	R	remaining 5-LO activity (% ± SEM at 1 µM)	IC_{50} [µM] or remaining 5-LO activity (% ± SEM at 10 µM)
117	3,4-diethoxybenzyl	96.1 ± 16.2	n.i. (91.5 ± 13.0)
118	2-pyridinylmethyl	85.3 ± 11.4	2.5
119	3-pyridinylmethyl	93.6 ± 20.5	2.8
120	4-pyridinylmethyl	74.1 ± 14.1	1.95
121	2-isoquinolylmethyl	112.6 ± 13.6	4.4
122	benzoyl	88.7 ± 6.0	10
123	2-acetoxybenzoyl	94.4 ± 2.9	6
124	3-chlorobenzoyl	92.2 ± 18.1	>10 (58.7 ± 9.5)
125	2-chlorobenzoyl	103.5 ± 11.3	> 10 (58.8 ± 6.5)
126	methyl acetate	86.0 ± 17.5	> 10 (56.7 ± 16.9)
127	2-carboxymethyl	135.0 ± 23.6	n.i. (85.5 ± 6.9)

Substitution of the N-benzyl in position 3 (**105-107**) resulted in notable loss of activity. Only the 3-fluoro-substituted derivative (**106**) was active in the range of

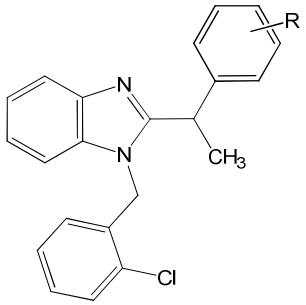
the para-substituted compounds (IC_{50} = 4.0 µM). A free hydroxy group at position 2 of the N-benzyl (**108**) lowered the IC_{50} to 4.5 µM. However, modifications of the hydroxy group by methylation (**109**) or acetylation (**110**) restored the potency. Especially acetylation at this site improved the inhibitory potency to 0.25 µM compared to **83**. Other small residues such as cyano (**111**), methyl (**112**) or fluoro groups (**113**) were well tolerated, yielding potent compounds with IC_{50} values from 0.45 µM to 0.7 µM. Introduction of two chloro substitutents at the N-benzyl lowered the potency around 9-fold for the 2,6-dichloro-substituted compound (**114**) compared to the 2,4-dichloro-derivative (**115**). Introduction of multiple alkoxy groups such as 3,4,5-trimethoxy (**116**) or 3,4-diethoxy (**117**) clearly impaired the potency. This result confirms that bulky substitutents are not tolerated at other positions than position 2. Exchange of the N-benzyl ring by a heterocyclic element such as pyridinylmethyl (**118-120**) or isoquinolylmethyl (**121**) resulted in still active compound, with IC_{50} values between 1.95 and 4 µM. Especially the bulky isoquinoline moiety further diminished the inhibitory potency. In the next step, a benzoyl group instead of N-benzyl was inserted into the molecule. This variation seemed to be ineffective, since the unsubstituted (**122**) as well as the 3-chlorobenzoyl (**124**) and 2-chlorobenzoyl (**125**) analogues of **83** showed an IC_{50} of 10 µM or higher. Solely, the 2-acetoxybenzoyl derivative (**123**) slightly restored the potency, underlining the preference of an acetoxy substitiution in position 2. Replacement of the aromatic substitutents by aliphatic residues such as methyl acetate (**126**) or the free carboxylic acid (**127**) failed to efficiently reduce 5-LO product formation.

All derivatives described so far were available as racemates. In order to examine the influence of stereochemical aspects on the efficacy, achiral analogues of the lead compound **(128, 129)** were synthesized in the group of Prof. Banoglu at the University of Ankara (**Table 23**).

Table 23 5-LO inhibition of achiral derivatives and with modified isobutyl structure.
PMNL were stimulated with 2.5 µM A23187; Data shown as % of remaining 5-LO activity at 1 µM (mean ± SEM) versus vehicle (0.3% DMSO), unless otherwise stated; n ≥ 3



#	R'= R''	remaining 5-LO activity (% ± SEM at 1 µM)	IC_{50} [µM]
128	-CH_3	33.3 ± 10.3	0.7
129	-H	25.6 ± 11.2	0.5



#	R	remaining 5-LO activity (% ± SEM at 1 µM)	IC_{50} [µM]
130	4-CH_3	80.9 ± 13.9	2.8
131	3-benzoyl	90.2 ± 2.3	3

When two methyl groups were present at the bridging carbon atom (**128**), the potency was decreased more than 2-fold compared to **83**, whereas replacement by two hydrogen atoms (**129**) had only marginal influence. Additionally, the role of the isobutyl moiety at the lead compound **83** was studied while replacing it by 4-methyl (**130**) or a 3-benzoyl group (**131**). However, these modifications

retained the activity, but failed to enhance it, suggesting a benefit for the isobutyl fingerprint of **83** (**Table 23**). BWA4C was used in all incubations at 0.3 µM as control inhibitor with 3.3 ± 1.3 % remaining activity. In addition to the aforementioned derivatives, another structural optimization was made by modifications at C-5 of the benzimidazole core. Especially, the 5-methoxy and 5-hydroxy derivative as well as substitution with 2-pyridinylmethyl at this site further enhanced the potency compared to **83** with IC_{50} values between 0.12 and 0.19 µM [257]. As described before, the lead compound **83** only weakly inhibited 5-LO under cell-free conditions. Accordingly, no inhibitory effects on semi-purified recombinant 5-LO were also found for selected potent compounds at 10 µM (data not shown).

To sum up, the benzimidazole scaffold was found to be suitable for the design of novel anti-LT drugs. In a docking study, an interaction with the binding site of FLAP could be demonstrated for the lead compound **83** as well as some analogues [257]. The anti-inflammatory properties of **83** *in vivo* were demonstrated in the carrageenan-induced pleurisy model in rats**.** Several chemical modifications at different sites of the molecule further improved the inhibitory efficiency *in vitro*. Therefore, the benzimidazole structure carrying an ibuprofen fingerprint was identified as new and useful tool for the development of new anti-LT agents.

4.5 Modified NSAIDs as dual inhibitors of 5-LO and mPGES-1

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for the treatment of pain, fever and inflammatory diseases. Their anti-inflammatory actions are based on the inhibition of prostaglandin and thromboxane formation [259]. However, the application of NSAIDs goes along with side-effects due to blockade of COX-1 and COX-2. Although the use of selective COX-2 inhibitors ("Coxibs") led to less gastrointestinal side effects compared to COX-1-inhibiting substances [260], a higher risk of cardiovascular events especially for rofecoxib was found [261]. In the last years, the inhibition of mPGES-1-derived PGE_2 formation has become an interesting approach for the treatment of

inflammatory diseases. Therefore, the dual inhibition of 5-LO and mPGES-1 is believed to have a broad anti-inflammatory efficiency and a lower rate of side effects [219].

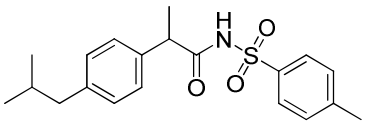
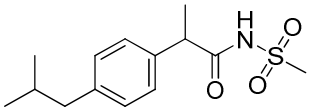
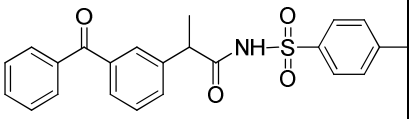
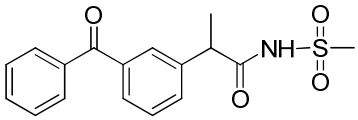
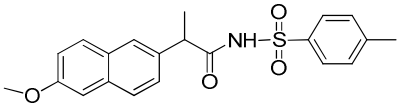
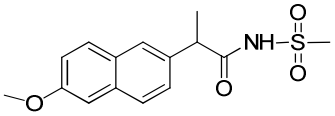
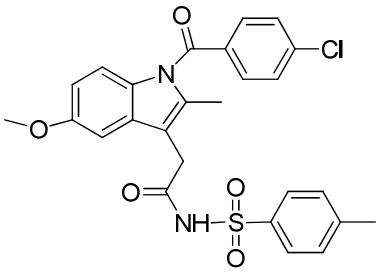
4.5.1 Rationale for selection of the test compounds

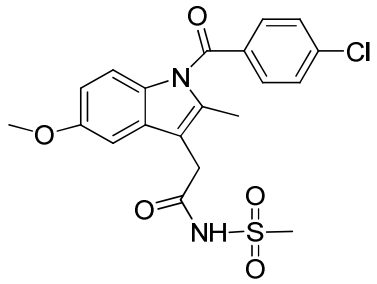
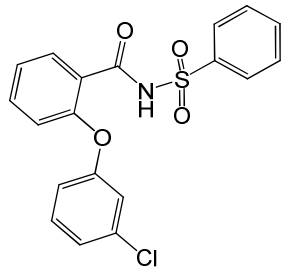
The present test compounds were synthesized by M. Elkady in the group of Prof. S. Laufer (University of Tübingen, Germany) (**Table 24**). The structures of the test compounds are based on common acidic NSAIDs such as ibuprofen (**132, 133**), ketoprofen (**134, 135**), naproxen (**136, 137**), indomethacine (**138, 139**), diclofenac (**140**) and lonazolac (**141-145**). The carboxylic acid function was synthetically modified by introducing a sulfonamide moiety. This modification was described before to improve the dual mPGES-1/5-LO inhibition for licofelone, a dual COX/LOX-inhibitor [262].

4.5.2 Effects of the compounds on 5-LO and mPGES-1 product formation

All compounds were screened at 10 µM for mPGES-1 inhibition in a cell-free assay using microsomes of A549 cells. Inhibition of 5-LO product formation was tested in A23187-stimulated human PMNL. Additionally, recombinant, semi-purified 5-LO was used to identify direct interactions of the compounds with 5-LO. BWA4C at 0.3 µM was used as control for 5-LO inhibition (IC_{50} = 0.16 µM and 0.04 µM in PMNL and semi-purified 5-LO, respectively) and 1 µM MK-886 acted as control for inhibition of mPGES-1 (IC_{50}= 2.3 µM).

Table 24 5-LO and mPGES-1 Inhibition of modified NSAIDs
PMNL were stimulated with 2.5 µM A23187, mPGES-1 activity was measured in microsomal preparations of A549 cells (20 µM PGH_2); Data shown as % of remaining enzyme activity at 10 µM (mean ± SEM) versus vehicle (0.3% DMSO) or (*) IC_{50} (µM ± SEM); n ≥ 3

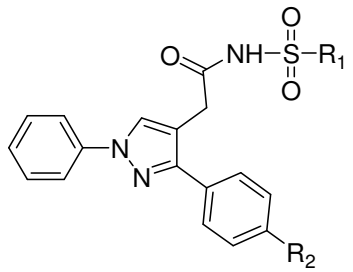
#	structure	mPGES-1 (% of control at 10 µM) (*) IC_{50} [µM]	5-LO PMNL (% of control at 10 µM) (*) IC_{50} [µM]	purified 5-LO (% of control at 10 µM)
132		78.6 ± 6.4	67.7 ± 8.9	86.8 ± 7.6
133		67.9 ± 15.8	91.2 ± 4.3	94.9 ± 23.0
134		76.3 ± 10	78.9 ± 12.7	95.8 ± 13.4
135		78.6 ± 2.1	90.7 ± 10.6	132.0 ± 10.7
136		72.8 ± 6.9	148.9 ± 26.9	102.1 ± 19.2
137		92.5 ± 4.8	109.8 ± 16.7	102.3 ± 3.6
138		30.2 ± 3.3 (*) 6.4 ± 1.4 µM	8.1 ± 8.1 (*) 2.9 ± 0.8 µM	76.2 ± 3.9

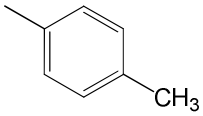
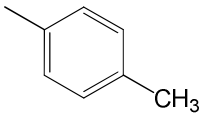
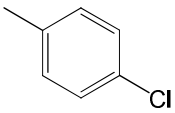
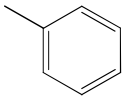
#	structure	mPGES-1 (% of control at 10 µM) (*) IC_{50} [µM]	5-LO PMNL (% of control at 10 µM) (*) IC_{50} [µM]	purified 5-LO (% of control at 10 µM)
139		73.6 ± 8.4	64.9 ± 10.3	92.9 ± 19.4
140		107.0 ± 15.0	72.2 ± 6.6	107.9 ± 20.0

Within this series, only the indomethacin derivative **138** inhibited mPGES-1 and 5-LO product formation remarkably (IC_{50}= 6.4 µM for mPGES-1 and 2.9 µM for 5-LO, respectively). In contrast, **139,** another indomethacine derivative, containing a methyl group instead of the more lipophilic tolyl (**138**), barely influenced 5-LO and mPGES-1 product formation at 10 µM. Indomethacin itself reduced neither 5-LO- nor mPGES-1 product formation at 10 µM (unpublished observations, not shown).

In addition, a second set of compounds was synthesized based on lonazolac (**Table 25**). Lonazolac, an arylacetic acid derivative was approved in the early 1980s and was used for the treatment of rheumatic diseases and pain. Anti-inflammatory properties of lonazolac were demonstrated *in vitro* by the inhibition of formation of LTB_4, its isomers as well as LTC_4 in PMNL and by the inhibition of histamine release in human basophils [263].

Table 25 5-LO and mPGES-1 Inhibition of lonazolac analogs
PMNL were stimulated with 2.5 µM A23187, mPGES-1 activity was measured in microsomal preparations of A549 cells (20 µM PGH_2); Data shown as % of remaining enzyme activity at 10 µM (mean ± SEM) versus vehicle (0.3% DMSO) or (*) IC_{50} (mean ± SEM); n ≥ 3



#	R1	R2	mPGES-1 (% of control at 10 µM) (*) IC_{50} [µM]	5-LO PMNL (% of control at 10 µM) (*) IC_{50} [µM]	purified 5-LO (% of control at 10 µM)
141		-Cl	26.6 ± 1.4 (*) 3.4 ± 0.8 µM	3.3 ± 2.8 (*) 2.5 ± 0.3 µM	65.3 ± 9.3
142		-phenyl	9.8 ± 1.7 (*) 1.7 ± 0.2 µM	0 ± 0 (*) 3.5 ± 1.5 µM	44.7 ± 13.7
143		-Cl	20.7 ± 3.0 (*) 2.3 ± 0.2 µM	0 ± 0 (*) 2.9 ± 1.1 µM	53.8 ± 14.0
144		-Cl	32.0 ± 2.5 (*) 5.9 ± 0.1 µM	27.6 ± 9.1 (*) 6.8 ± 2.3 µM	93.2 ± 4.9
145	$-CH_3$	-Cl	98.0 ± 26.9	70.7 ± 10.9	43.9 ± 16.2

The presence of a lipophilic, aromatic residue at the sulfonamide appeared to be necessary for dual inhibitory action. Substitutents such as p-tolyl (**141, 142**) or a 4-chlorophenyl (**143**) were advantageous compared to an unsubstituted phenyl moiety (**144**), leading to a potency towards mPGES-1 in the range of MK-886. Replacement of the aromatic moiety by a methyl group (**145**) strongly impaired the potency towards mPGES-1 as well as 5-LO. The best inhibitory potency for mPGES-1 was found when a biphenyl moiety (**142**) was present at the pyrazole,

resulting in an IC_{50} of 1.7 µM. However, nearly equal potency for both enzymes was realised with compound **143**, underlining that the chlorophenyl substitution at the sulfonamide moiety is favorable for dual mPGES-1/5-LO inhibition compared to the p-tolyl moiety of **141**. Recombinant, semi-purified 5-LO was not or only hardly inhibited by the compounds with IC_{50} values around 10 µM or higher.

Further, no inhibition of COX-1 was observed for selected compounds, as the introduction of the sulfonamide moiety seems to lead to a loss of COX-1 inhibition (data not shown). Introduction of a phenyl acetylene moiety at the indomethacin and lonazolac scaffold led to compounds with improved potency especially towards mPGES-1 [264]. The results suggest that structurally modified NSAIDs, especially based on the structure of lonazolac, may act as new leads for the development of dual inhibitors of 5-LO and mPGES-1.

5 Discussion

The investigation of a candidate compound requires suitable assay conditions to determine the effect on 5-LO product formation. Numerous publications describe the *in vitro* screening of 5-LO-inhibiting compounds. However, in some studies the physiological concentration of AA is not considered and therefore AA concentrations up to the milllimolar range or high concentrations of the compounds that cannot be reached *in vivo* are used. Additional assay conditions that should be critically assessed include the type and concentration of the applied stimuli, the used cell type as well as recombinant non-human-LOs (e.g. mouse or rat) where the regulation of catalytic 5-LO activity might differ. A reliable system for the *in vitro* screening of 5-LO inhibitors is the combination of a cell-free assay with recombinant 5-LO or cell homogenates and a cell-based test system using PMNL or monocytes from human blood. The cell-free assay allows the identification of direct interactions between the enzyme and the test compound. In contrast, the cell-based assay provides information on the impact of a candidate compound to aspects that regulate cellular 5-LO activity such as LOOH, FLAP, CLP, kinases, membrane association or inhibition of associated enzymes ($cPLA_2$, LTA4H) leading to reduced LT biosynthesis.

5.1 5-Hydroxy-indole-carboxylates as 5-LO inhibitors

Based on the results of a previous study by Landwehr et al., which described 2-amino-5-hydroxy-indole carboxylates as 5-LO inhibitors [236], the indole core was synthetically modified at different positions and SAR studies were undertaken. The presence of a hydroxy group at C-5 of the indole core enhanced the inhibitory potency of the compounds probably by its anti-oxidative property which keeps the catalytic iron of 5-LO in the Fe^{2+} state and thus prevents LT formation. Nevertheless, 2-amino-indole-methyl-carboxylate derivatives devoid of the 5-hydroxy moiety inhibited 5-LO product formation moderately with IC_{50} values from 2.4 µM to 12.5 µM in intact cells and in homogenates of E. coli with recombinant 5-LO. The SAR studies further indicated that the amino group

in position 2 of the indole is not necessary for potent inhibition in intact cells and thus substitution with an alkyl chain and linkage with a halogen-substituted phenyl moiety was advantageous.

The largest improvement of the potency was achieved by anellation of a benzene to the indole yielding a benzo[g]indole carboxylate backbone. Variations at the esterified moiety at C-3, leading to voluminous benzyl carboxylate derivatives resulted in comparable IC_{50} values to the corresponding ethyl carboxylate derivatives. Substituents at N-1 led to reduced potency or total inactivity of the compounds especially in the cell-based assay, suggesting a hindered uptake in to the cells. Anellation of methoxybenzene, pyridine or introduction of a biphenyl residue to the indole backbone could not further improve the potency of the compounds. Especially modifications or replacement of the hydroxy group at C-5 strongly reduced the potency, underlining the important role of this moiety for the inhibitory action. Finally, the 3-chlorobenzyl-substituted benzo[g]indole-3-carboxylate **8a** emerged as the most active compound in the cell-based as well as in the cell-free assay. Further *in vitro* experiments characterized compound **8a** as a direct, non-competitive and reversible 5-LO inhibitor. A whole blood assay and an animal model of LT-mediated inflammation, both models resembling *in vivo* conditions, confirmed the anti-inflammatory actions of **8a** [239]. In addition, studies by A. Koeberle in our workgroup revealed that some representatives of the benzo[g]indole-carboxylates inhibit mPGES-1-derived product formation which further expands the anti-inflammatory spectrum of these compounds [230].

5.2 Substituted 1,4-benzoquinones and related polyphenolic compounds that inhibit 5-LO product formation

Polyphenolic compounds are able to interfere with the active site iron of 5-LO and interrupt the catalytic cycle or act by iron-chelating properties of the hydroxy groups and thus reduce 5-LO product formation.

Within the polyphenolic compounds, the catechol derivatives **18a** and **18b** showed a considerable inhibitory efficacy with IC_{50} values of 130 nM and 76 nM towards recombinant 5-LO. The presence of two hydroxy groups and the high radical scavenging properties as observed in the DPPH assay suggest a redox-type mode for 5-LO inhibition. Accordingly, the presence of methoxy groups at **19a** instead of hydroxy groups completely abolished the potency towards recombinant 5-LO compared to the corresponding derivative **18a**. Surprisingly, although a redox-type mechanism seems likely, no significant inhibition of p12-LO and 15-LO product formation was observed for the polyphenolic compounds suggesting a selective interference with 5-LO [249].

A predominant reducing intracellular environment makes the 1,4-benzoquinone structure susceptible to reductive conversion to the diphenolic 1,4-hydroquinone which then acts as redox-active or iron-ligand inhibitor of 5-LO product formation. Interestingly, some of the investigated 1,4-benzoquinones were highly potent in the cell-free assay excluding any metabolic change and rather suggests a direct interaction with 5-LO. Indeed, molecular docking studies showed a direct interference of the compounds with 5-LO via hydrogen bonds and π-π interactions. Moreover, the inhibitory potencies obtained from the *in vitro* assays correlate with the observation from the docking studies that the 1,4-benzoquinone series forms more stable interactions with 5-LO than the resorcinol compounds [249].

Within the 1,4-benzoquinone series, the decahydronaphthyl-substituted derivative **16d** turned out to be very potent in human neutrophils (IC_{50}= 0.58 µM). In contrast, the cyclohexyl-substituted compound **16c** could not reduce LT formation in both assays, demonstrating that the inhibitory potency of the compounds is determined by the lipophilic substitutent at the benzoquinone core. The DPPH assay showed that **16d** itself has no radical scavenging properties and thus the cellular environment is required for its inhibitory action. In cell-free assays with recombinant 5-LO and cell homogenates the efficacy of **16d** was much weaker compared to intact cells. Further experiments revealed a

correlation between an elevated oxidative tone and the reduced inhibitory potency of **16d** [248]. Therefore, the inhibitory mechanism of **16d** is comparable to non-redox-type inhibitors such as ZM230487 or CJ-13,610 [139] and not based on interference with the catalytic redox cycle of 5-LO. p12-LO and 15-LO product formation were not affected significantly by **16d** or any of the other 1,4-benzoquinones at 10 µM which further supports an inhibitory mechanism independently from the redox cycle of 5-LO catalysis. Moreover, **16d** failed to inhibit mPGES-1 or COX-1 activity and COX-2-derived 6-keto-$PGF_{1\alpha}$ was blocked with 12-fold lower potency (IC_{50} = 7.3 µM) than 5-LO in neutrophils (IC_{50}= 0.58 µM). In addition, the *in vivo* potency of **16d** was demonstrated in the whole blood assay and in two animal models, where further anti-inflammatory actions were identified [248]. In a recent study, the 2,5-dihydroxy-1,4-benzoquinone embelin was identified as potent dual inhibitor of 5-LO and mPGES-1 [265] which encourages to further investigations of the 1,4-benzoquinones for the development of anti-inflammatory drugs.

5.3 Pyrazol-propanoic acid derivatives as inhibitors of LT biosynthesis

Within a series of 1,5-diaryl-substituted pyrazol-propanoic acid amide and ester derivatives, 4 compounds reduced LT formation significantly at 10 µM in PMNL after stimulation with A23187 and 20 µM AA. In order to define SAR, structural modifications of the 4 identified derivatives such as introduction of different aryl substituents at N-1 or N-2 of the central pyrazole, shortening of the propanoic acid side-chain and different substituents in para-position on C5-phenyl of the pyrazole were made. The replacement of the chloropyridazine moiety at N-1 of the pyrazole by other aryl substituents such as phenyl, isoquinoline or benzyl and shortening of the side-chain yielded compounds with only weak or no efficacy towards recombinant 5-LO. In the cell-based assay, especially the phenyl- and isoquinoline-substituted compounds reduced LT formation significantly. Further structural variations at the tert-butyl-piperazine-substituted compound **33** by introduction of different aryl substituents at N-1 of the pyrazole and variations at the para-position of the phenyl ring resulted in

compounds with IC_{50} values between 1.8 µM to 8 µM in the cell-based assay. Notably, all pyrazol-propanoic acid derivatives showed weak ($IC_{50} \geq 10$ µM) or no efficiency in the cell-free assays with recombinant 5-LO, suggesting a point of attack at key steps of cellular 5-LO activity.

In the next step, 9 compounds with different moieties at C-5 and N-1 of the pyrazole core were chosen for investigations on the pharmacological profile. With regard to the efficacy of the compounds, modifications of the substrate concentration resulted in heterogeneous outcomes and were not directly related to the structural properties at C-5 and N-1. Thus, the efficacy of the tert-butyl-benzylpiperazine-substituted derivatives **33** and **44** was unaffected by the substrate concentration, whereas the corresponding isoquinolyl derivative **48** and the chloropyridazinyl-substituted compounds **29** and **36** reduced LT biosynthesis only when exogenous AA was supplied, suggesting a competitive mode of inhibition. High amounts of AA perhaps bind to the regulatory site of 5-LO and facilitate the interference of the pyrazol-propanoic acids with the catalytic activity. In contrast, the impaired efficacy when high amounts of AA were present as observed for the phenyl-substituted derivatives **45** and **46** as well as the remaining product formation at inhibitor concentrations > 10 µM was described for FLAP inhibiting-compounds such as licofelone or MK-886 [238]. The increase of LT formation at concentrations > 3 µM of compound **50** may be attributed to the formation of micelles consisting of AA and the compound leading to impaired access to the enzyme, since this aspect occurred only at high concentrations of exogenous AA in the assay.

Previous studies showed that the efficacy of non-redox-type inhibitors depends on the activation pathway of 5-LO. Osmotic cell stress such as hypertonic NaCl activates p38 MAPK and associated downstream kinases in a calcium-independent manner leading to 5-LO activity [126]. Thus, cell-stress-induced product formation strongly impaired the potency of the non-redox-type inhibitors ZM230487 and L-739.010, but not of the iron-ligand inhibitor BWA4C [200]. While for **44** the inhibitory potency was unaffected by the

activation pathway of 5-LO, cell-stress-induced 5-LO activation strongly reduced the potency of **33**. Obviously, the calcium-dependent activation of 5-LO, but not the phosphorylation-dependent activation contributes to the inhibition of LT biosynthesis. Cell-stress-induced 5-LO product formation was also reduced by **45** and **46**, but in the same manner as after stimulation with A23187 together with 40 µM AA (not shown). Taking into account that elevated AA concentrations and cell-stress-induced activation of 5-LO impaired the potency of **45** and **46**, these compounds share also some *in vitro* chararacteristics of non-redox-type inhibitors. In contrast, the inhibitory effect of compound **48** was dependent on the phosphorylation-dependent activation of 5-LO caused by cell-stress and high amounts of AA. For some compounds (**29, 36**) neither stimulation with A23187 alone nor stimulation by cell-stress led to reduced 5-LO product formation, showing that the amount of exogenous AA influences the inhibitory effect in any way.

The catalytic activity of 5-LO is determined by the activity of glutathione peroxidases which control the concentration of LOOH in the cell [136] and the *in vitro* potency of non-redox-type inhibitors was shown to depend on the cellular peroxide levels [139], [201]. However, addition of GSH to homogenates of PMNL in order to restore peroxidase activity and thus reduce the LOOH concentration had no influence on the efficacy of the chosen pyrazol-propanoic acid derivatives. These data clearly show that the compounds reduce 5-LO product formation independently from the cellular LOOH concentration and act in a different manner other than non-redox type inhibitors.

The efficacy of the pyrazol-propanoic acid derivatives, was much higher in intact PMNL than in the cell-free assays using recombinant 5-LO. Interestingly, for compound **33** and **48** the inhibitory effect was significantly stronger in 40,000 x g homogenates of E. Coli compared to semi-purified recombinant 5-LO protein, suggesting that remaining lipids or other cellular components improve the binding of the compounds to 5-LO.

The first step of LT biosynthesis requires the release of endogenous AA by $cPLA_2$. The strong inhibitory effect of the phenyl- and isoquinolyl-substituted compounds **44** and **48** in the cell-free assay with recombinant $cPLA_2$ was not confirmed in the cell-based assay by the measurement of AA-release in human monocytes. Moreover, compound **48** reduced 5-LO product formation in PMNL only after addition of exogenous AA which circumvents $cPLA_2$ activation, but not upon stimulation with A23187 alone. These results question an interference with $cPLA_2$ as mode of action for the inhibition of LT biosynthesis.

Celluar 5-LO activity is not strictly dependent on the presence of intra- or extracellular calcium, since addition of exogenous AA to Ca^{2+}-depleted PMNL induces 5-LO product formation accompanied by a rapid and strong phosphorylation of ERK 1/2 and p38 MAPK [143]. Interestingly, the reduced 5-LO product formation in calcium-depleted PMNL of the tert-butyl-piperazine-substituted derivatives **33** and **48** and to a lower extent of **44** suggests an interference with calcium-independent mechanisms of 5-LO activity. AA and some lipoxygenase-derived metabolites can stimulate further neutrophil functions such as degranulation, ROS generation or activate other kinases such as PKC or PI3K [266] and hence interactions of the compounds with these signaling pathways seem possible.

A concomitant inhibition of LT and PG formation by interference with 5-LO and COX represents an efficient anti-inflammatory strategy by lowering the gastrointestinal and cardiovascular side effects of the classical NSAIDs. Accordingly, the observed strong inhibition of COX-1-derived 12-HHT formation in human platelets of the phenyl- and quinoline-substituted pyrazol-propanoic acid derivatives results in an enhanced anti-inflammatory effect of these compounds. Considering the chemical structures, especially a phenyl or quinoline moiety at N-2 of the pyrazole in combination with the tert-butyl-benzyl-piperazine (**44, 48**) or the isopropyl-phenyl-propanoate moieties (**36, 50**) favours a dual inhibition of 5-LO and COX-1 product formation. Moreover, the

formation of COX-2 and mPGES-1-derived 6-keto-$PGF_{1\alpha}$ and PGE_2 was not influenced by the compounds.

The C2 domain of 5-LO mediates the calcium-dependent translocation of cytosolic 5-LO to the nuclear membrane [121]. An influence on the subcellular redistribution of 5-LO may lead to inadequate catalytic activity and reduced LT formation. Compared to the control compound hyperforin which potently reversed the binding to the nuclear membrane, some of the pyrazol-propanoic acid derivatives slightly increased the amount of 5-LO in the nuclear fraction, whereas others had no effect on the intracellular 5-LO distribution. An accumulation of 5-LO at the nuclear membrane caused by the compound may lead to reduced LT formation by anchoring the enzyme via the C2-domain at the membrane yielding in a modified orientation towards FLAP which impairs the conversion of AA. However, the accumulating effect in the nuclear fraction occurred at high concentrations of 30 µM of the compounds which points to a minor reason for the inhibition of LT biosynthesis in PMNL.

Phosphorylation of 5-LO by ERK2 and the p38 MAPK-dependent MK2 is an important factor for AA-induced product formation in PMNL [267]. While an influence of the pyrazol-propanoic acid derivatives on the phosphorylation state of ERK2 was not apparent, **44** and **48** induced a strong phosphorylation of p38 MAPK in fMLP-stimulated PMNL. This observed effect may be caused by an interference of the compounds with MAPK dephosphatases (MKP) [268], [269]. Accordingly, the hyperactivation of p38 MAPK as observed in MKP-deficient cells [270] leads to a loss of the kinase function and thus no 5-LO phosphorylation by the downstream kinase MK2. However, it has to be noted that the enhanced phosphorylation of p38 MAPK might be caused by the compounds itself without the need of activation of the cells by fMLP. Interestingly, the natural compound tryptanthrin which inhibits cellular LT biosynthesis also provokes enhanced p38 MAPK phosphorylation without affecting ERK phosphorylation [271].

In addition, the efficacy of a compound in whole blood provides important informations about the *in vivo* behaviour. Therefore, the potency of a compound may be altered by plasma protein binding, fatty acids, phopholipids or other factors present in the blood plasma compared to its *in vitro* efficacy. Unfortunately, the pyrazol-propanoic acids had no effect on 5-LO product formation in the plasma of whole blood up to 10 and 30 µM, respectively. Due to the lipophilic character of the compounds, a binding to plasma proteins or other components leading to impaired efficacy seems likely. Nevertheless, investigations in animal-based models might reveal other anti-inflammatory actions of the compounds.

In total, the exact mode of LT biosynthesis inhibition of the pyrazol-propanoic acid derivatives remains unknown. *In vitro* experiments revealed no redox-active or iron-ligand properties which can be further excluded by the inactivity towards recombinant 5-LO. Taken together, the pyrazol-propanoic acid compounds influence cellular LT biosynthesis in various ways which cannot be readily explained. With regard to the need of an intact cellular environment one might speculate about interactions of some representatives with FLAP, CLP or binding to the regulatory binding site of the enzyme and possible competition with phospholipids or calcium at the C2 domain of 5-LO. In addition, the dual COX-1/5-LO inhibitory properties of certain derivatives, the class of the pyrazol-propanoic acid derivatives provide a good basis for the further development of anti-inflammatory compounds.

5.4 Identification of LT biosynthesis inhibitors by virtual screening

The use of computer-aided structure- and ligand based strategies in the drug design of 5-LO inhibitors led to the identification of new lead molecules [272]. Here, a combined structure- and ligand-based virtual screening approach was used for the identification of novel chemical scaffolds with inhibitory activity on cellular LT biosynthesis. The applied strategy involved the collection of diverse known FLAP-inhibiting compounds followed by the generation of a ligand-based pharmacophore model and virtual screening of a compound database,

subsequent refinement by docking and final selection of the test compounds based on chemical and literature knowledge [257]. Finally, a subset of 8 structurally heterogenous compounds was tested for the ability to inhibit 5-LO product formation, whereof 4 compounds clearly reduced 5-LO activity in a cell-based assay using PMNL and in a cell-free assay with recombinant 5-LO at a concentration of 10 µM, suggesting a direct inhibition of 5-LO.

The diarylmethylisoxazole compound **78** was identified as a potent and selective direct 5-LO inhibitor without significant effects on 12-LO and COX-1 product formation. Subsequent structural modifications of compound **78** indicated that the presence of an ethyl carboxylate at the isoxazole structure completely abolished the inhibitory potency, whereas the corresponding free acids tolerated alterations of the substituents at the aromatic rings of the molecule. Especially the presence of fluorine at the benzyloxy moiety (**89, 92**) improved the potency of the resulting 4,5-diarylisoxazole carboxylic acid derivatives in the cell-based assay around 3-fold compared to the lead compound **78.** Under cell-free conditions with recombinant 5-LO, the IC_{50} values for the diarylisoxazole-carboxylic acids were about twice lower compared to intact PMNL, suggesting a reduced inhibitory effect in cellular environment due to hindered uptake or attenuation by intracellular factors.

On the other hand, the benzimidazole derivative **83** identified in the virtual screening almost completey blocked LT formation in intact PMNL (IC_{50}= 0.31 µM), but had weak influence on 5-LO activity under cell-free conditions. The fact that high amounts of exogenous AA impaired the potency and the weak efficacy of **83** in cell-free systems suggest FLAP as possible point of attack. Moreover, the apparent structural similarity of **83** with licofelone or MK-886 as well as the absence of redox and iron-ligand properties and no influence of reducing conditions in the cell-free assay lets FLAP therefore appear as potential target.

Several compounds such as the FLAP inhibitor MK-886 [165], licofelone [238] or the natural compound hyperforin [204] were described to interfere with the translocation of 5-LO. Stimulation of PMNL with A23187 induces the movement from the cytosol to the nuclear membrane, where conversion of AA to LTs occurs with the participation of FLAP and $cPLA_2$ [252]. In fact, **83** partly reversed 5-LO translocation to the nuclear membrane from the concentration of 1 µM. However, a complete reversion was not achieved up to 10 µM. Further studies using immunofluorescence microscopy revealed that inhibition of 5-LO translocation does not necessarily correlate to the potency of a compound inLT suppression (observations by C. Pergola).

Structural variations of the lead compound **83** were tolerated best in 2-position of the N-benzyl group with the exception of a free hydroxyl group, suggesting that hydrogen bond forming and/or the general increased polarity impairs the efficacy. Interestingly, a 2-acetoxybenzyl substituent enhanced the efficacy resulting in an IC_{50} value of 0.25 µM. On the other hand, substituents in 3- or 4-position of the N-benzyl as well as multiple substituents mainly reduced the potency except the 2,4-dichlorobenzyl moiety. Moreover, the replacement of the N-benzyl moiety by heterocyclic rings caused a strong or total loss of inhibitory activity as in the case of N-benzoyl substitution (**122-125**) or alkyl carboxylic acids and esters (**126, 127**). Together, other substituents than 2-substituted N-benzyl appeared to be detrimental for potent inhibition of 5-LO product formation in PMNL. A slight decrease of the potency was also apparent after preparation of achiral derivatives by addition or complete removal of methyl groups on the chiral carbon of **83**, proving that the inhibitory action of the racemic mixture is sufficient. Moreover, the isobutyl structure at the upper part of the lead molecule was found to be favorable, whereas the inhibitory potency was diminished around 10-fold when a methyl (**130**) or benzoyl group (**131**) was added. The resulting isobutylphenylethyl fingerprint, resembling the structure of ibuprofen might contribute to the anti-inflammatory effect of the benzimidazole compounds.

Further investigations included docking studies with the lead compound **83** and some analogues, where direct interferences of the compounds with the protein structure of FLAP could be shown [257]. Additional protein pull-down assays using derivatized **83** linked to an unsoluble matrix confirmed FLAP as a direct binding partner of **83** [273].

In the human whole blood assay, which reflects more physiological conditions, **83** suppressed LT formation upon stimulation with A23187 or LPS/fMLP with IC_{50} values of 10.1 µM and 3-6 µM [274], respectively. Additional anti-inflammatory effectiveness of **83** could be demonstrated in the carrageenan-induced pleurisy in rats and the zymosan-induced peritonitis in mice, two established *in vivo* models of acute inflammation. In both animal models, the amount of LTB_4, LTC_4 and the number of inflammatory cells was significantly reduced by **83**, although not to the same extent as by the reference compound MK-886 [273].

Taken together, the applied virtual screening approach using a combined ligand- and structure-based pharmacophore model led to the identification of potent 5-LO inhibitors. 4,5-diarylisoxazole derivatives were characterized as potent, direct 5-LO inhibitors with IC_{50} values in the low micromolar range. Moreover, the identification of the derivative **83** proposes the benzimidazole scaffold as new chemotype for LT biosynthesis inhibitors targeting FLAP. The potent and selective inhibition of 5-LO product formation of **83** without affecting 12- or 15-LOs, COX-1/-2 or mPGES-1 and the demonstrated *in vivo* efficacy [257, 273] offers considerable potential for the further development of FLAP inhibitors.

5.5 Derivatives of NSAIDs as dual 5-LO/mPGES-inhibitors

Over time, concomitant inhibition of PG and LT biosynthesis turned out as alternative approach for anti-inflammatory therapy. The blockade of COX enzymes shifts AA metabolism towards 5-LO-derived LT formation. Since besides inhibition of COX, elevated LT levels seem also lead to gastric damage, the dual inhibition of PG and LT formation appears reasonable [219].

Furthermore, a selective inhibition of mPGES-1-derived PGE_2 formation seems to be superior over inhibition of the COX enzymes and may reduce side-effects of classical NSAIDs [30]. Together, a dual inhibition of mPGES-1 and 5-LO product formation promises broader anti-inflammatory efficacy and reduced gastrointestinal and cardiovascular side-effects.

In fact, the modification of the carboxylic acid function with a sulfonamide group substituted with an aromatic moiety generated derivatives of the NSAIDs indomethacine and lonazolac which potently inhibited 5-LO and mPGES-1 product formation (**138, 141-144**). The presence of a bulky and lipophilic, aromatic substituent such as phenyl, tolyl or chlorophenyl at the sulfonamide was found to be necessary, since the inhibitory potency towards both enzymes was abolished with a methyl group. Regarding mPGES-1 inhibition, a biphenylic moiety at the lonazolac derivative **142** improved the potency 2-fold compared to the 4-chlorophenyl substituted **143,** but the efficacy towards 5-LO was slightly reduced. An adequate concomitant inhibition of 5-LO and mPGES-1 was achieved with compound **143** (IC_{50} =2.9 µM for 5-LO and IC_{50} = 2.3 µM for mPGES-1), suggesting that the lipophilic 4-chlorophenyl moiety favors the dual inhibition. Regarding COX-1 inhibition, for **138** and **142** no significant effect on 12-HHT formation was observed at 10 µM in a cell-free assay, proving that the structural modification with the sulfonamide moiety, shifts the anti-inflammatory effect from COX-1 inhibition towards 5-LO and mPGES-1 inhibition. Further structural modifications of the indomethacin derivatives improved the potency compared to compound **138** (IC_{50}= 2.9 µM for 5-LO and IC_{50}= 6.4 µM for mPGES-1) up to IC_{50} values of 1.75 ± 0.6 µM and 0.9 ± 0.4 µM for mPGES-1 and 5-LO, respectively [264].

Taken together, the replacement of the carboxylic acid function of the NSAIDs indomethacine and lonazolac by a sulfonamide moiety leads to a loss of COX-1 inhibition and enhances the inhibitory potency towards 5-LO and mPGES-1.

6 Summary

Leukotrienes (LTs) represent important lipid mediators derived from the PUFA arachidonic acid (AA) with broad involvement in pathophysiological conditions. 5-LO catalyzes the first two steps of LT formation from AA which is released from membrane phospholipids by $cPLA_2$. In a first step, molecular oxygen is incorporated into AA by 5-LO resulting in 5-H(P)ETE followed by subsequent dehydration yielding the epoxide intermediate LTA_4. Further enzymatic conversion of LTA_4 by LTA4H and LTC4H results in the formation of LTB_4 and cysteinyl-LTs. LTs are associated with many diseases such as asthma, atherosclerosis, osteoporosis, cardiovascular diseases as well as certain types of cancer. Therefore, specific pharmacological intervention with LT synthesis is a promising strategy for the treatment of these diseases.

The complex regulation of the catalytic activity of 5-LO allows various points of attack to inhibit LT formation. The cellular activity of the enzyme is regulated by several factors such as Ca^{2+}, MAPK, phospholipids such as PC, glycerides and CLP. Furthermore, 5-LO activation depends on the presence of LOOH for the oxidation of the active site iron from the ferrous (Fe^{2+}) to the ferric state (Fe^{3+}).

Hence, several pharmacological strategies are possible to intervene with 5-LO-derived LT formation. Beside the direct inhibition of 5-LO, antagonism at the LT and Cys-LT receptors, inhibition of FLAP or other enzymes of the AA-pathway ($cPLA_2$, LTA4H, LTC4S) as well as interference with other key steps of LT formation is conceivable. For example, FLAP inhibitors inhibit the transfer of AA and reduce the availibility of AA as substrate for 5-LO. Direct 5-LO inhibitors are classified into three types: (I) redox-type inhibitors that reduce the active-site iron und thus uncouple the catalytic cycle, (II) iron-ligand inhibitors that chelate the active-site iron and (III) non-redox-type inhibitors that compete with AA or LOOH. In addition, a novel, structurally heterogenous class of compounds with different or unknown mode of action was defined [202].

However, most of the potential drug candidates failed due to severe side effects such as methemoglobin formation or other toxic effects, unspecific action or were ineffective under *in vivo* conditions. So far, only the iron-ligand inhibitor zileuton and Cys-LT receptor antagonists entered the market for the treatment of asthma and allergic diseases. Therefore, alternative concepts and novel lead structures for the therapy of LT-related diseases are required.

In this study, different novel classes of LT biosynthesis inhibitors were identified. Since the efficacy of a test compound strongly depends on the assay conditions, detailed studies were performed in order to characterize the mechanistic aspects of 5-LO inhibition.

Based on a previous study by Landwehr et al [236], compound **1a** (methyl 2-(3-chlorophenylamino)-5-hydroxy-1H-indole-3-carboxylate) was identified within a series of indole-3-carboxylates as potent 5-LO inhibitor in intact cells (IC_{50}= 2.4 µM) as well as in a cell-free assay using recombinant 5-LO (IC_{50}= 0.3 µM). Structural modification of the indole-3-carboxylate core led to a series of benzo[g]indole-3-carboxylates with improved inhibitory potency. Subsequent investigations characterized compound **8a** (ethyl 2-(3-chlorobenzyl)-5-hydroxy-1H-benzo[g]indole-3-carboxylate) as a direct, non-competitive and reversible 5-LO inhibitor (IC_{50}= 0.23 µM in intact cells and IC_{50}= 0.03-0.1 µM in the cell-free assay). Furthermore, **8a** reduced 5-LO product formation in the human whole blood assay (IC_{50}= 1.6 µM) and showed anti-inflammatory actions *in vivo* in the carrageenan-induced pleurisy model in rats with comparable potency to the well-known iron-ligand inhibitor zileuton. Additional studies by A. Koeberle in our workgroup further characterized the benzo[g] indole-3-carboxylates as potent dual inhibitors of 5-LO and mPGES-1 without significant effects on COX enzymes. In this context, the 2-chlorophenyl-substituted derivative **8b** was the most potent compound (IC_{50}= 0.1 µM for mPGES-1 and 1.2 µM for 5-LO) among the benzo[g]indole-3-carboxylates [230].

SAR studies with 1,4-benzoquinone and respective polyphenolic derivatives revealed that the type of the substituent at the 1,4-benzoquinone or polyphenolic backbone determines the overall potency. Especially bulky groups such as naphthyl or dibenzofuranyl substituents favor the inhibition of 5-LO product formation. Surprisingly, some 1,4-benzoquinone compounds directly inhibited recombinant 5-LO, hence excluding bioactivation to the 1,4-diphenol structure and suggesting an inhibitory mode other than redox activity. Since 12- and 15-LO product formation were not significantly affected by selected potent compounds, an interference with the common redox cycle of the mammalian LOs can be excluded. Interestingly, the i*n vitro* inhibitory potencies correlated with the results from molecular docking studies, where more stable interactions of the 1,4-benzoquinone derivatives with 5-LO were found than for the polyphenolic compounds [249]. The 1,4-benzoquinone **16d** showed high inhibitory efficacy in PMNL (IC_{50}= 0.58 µM), but was less active in neutrophil homogenates and recombinant 5-LO (IC_{50}=11 µM). No radical scavenging properties of **16d** itself were apparent, but seemingly the reducing intracellular conditions are required for its inhibitory action. $cPLA_2$, COX-1 and mPGES-1 were not affected, whereas **16d** inhibited COX-2, but with weaker potency (IC_{50}= 7.3 µM) than 5-LO [248]. In the human whole blood assay, resembling *in vivo* conditions, **16d** and the catechol derivative **18b** were active (IC_{50}= 9.1 µM and 8-9 µM, respectively) and more anti-inflammatory efficacy of **16d** was demonstrated *in vivo* in two established animal inflammation models [248].

Following SAR studies with pyrazol-propanoic acid derivatives, 9 derivatives with different substituents at C-5 and N-1 of the pyrazole scaffold were chosen for mechanistic investigations. In general, the inhibitory potency of the compounds was higher in intact cells than in cell-free systems such as PMNL homogenates or recombinant 5-LO. For 2 compounds (**33, 48**) a stronger inhibitory effect was observed in homogenates of E. coli expressing recombinant 5-LO, whereas semi-purified recombinant 5-LO was not affected, indicating an influence of cellular components on the efficacy. Heterogenous mechanistic properties were observed at varying substrate concentrations or

different celluar 5-LO activation by calcium or osmotic cell stress. Interestingly, some phenyl- and quinoline-substituted compounds (**44, 46, 48-50**) potently reduced COX-1-derived 12-HHT formation in platelets (IC_{50}= 1.8 µM for **44**, 0.3 µM for **46** and 0.014-1 µM for **48-50**, respectively) without effects on COX-2 and mPGES-1 product formation. Thus, the dual 5-LO/COX-1 inhibition expands the anti-inflammatory effectiveness of the compounds.The observed effects of some compounds on the subcellular distribution of 5-LO required high concentrations *in vitro* and the extent was not comparable to the positive control hyperforin. Upon stimulation with fMLP, the compounds **44** and **46** caused a strong upregulation of p38 MAPK phosphorylation in PMNL, but this effect cannot clearly explain the inhibitory action. In total, the pyrazol-propanoic acid derivatives are inhibitors of cellular 5-LO product formation and partly inhibit COX-1-derived PG formation.

The use of a combined ligand- and structure-based pharmacophore model yielded a set of 8 structurally diverse compounds (**Table 19**).

The 4,5-diarylisoxazole derivative **78** inhibited LT formation in A23187-stimulated PMNL (IC_{50}= 4.4 µM) and in semi-purified 5-LO to around 40 % at 30 µM. Inclusion of 40 µM exogenous AA lowered the inhibitory potency in PMNL (IC_{50}= 6 µM). The presence of flourine at the outer phenyl ring and replacement of a methyl group by carboxylic acid function at the isoxazole core (**89, 92**) enhanced the potency about 3-fold in the cell-based assay and almost 5-fold towards recombinant 5-LO, respectively.

In contrast, the benzimidazole derivative **83** potently reduced A23187-induced LT formation in PMNL (IC_{50}= 0.31 µM), but under cell–free conditions, the inhibitory potency was nearly absent. Since mechanistic properties of non-redox-type inhibitors and radical scavenging properties could be excluded, FLAP was investigated as potential target of **83**. In fact, the interaction of **83** and related derivatives with FLAP was confirmed in docking studies and protein fishing experiments using immobilized compound [257], [273]. Moreover, **83**

partly reversed 5-LO translocation, a fact which was described for other FLAP inhibitors before. Subsequent structural modifications and SAR studies based on **83** yielded more potent analogues [257], disclosing the benzimidazole scaffold as new chemotype for further development of anti-LT drugs.

The simultaneous inhibition of LT and PG biosynthesis is supposed to lead to better gastrointestinal tolerability and anti-inflammatory efficacy. Therefore, dual 5-LO/mPGES-1 inhibitors promise a good alternative to NSAIDs. Structural modification of common NSAIDs by introduction of a sulfonamide scaffold connnected with a lipophilic aromatic substituent led to potent dual 5-LO/mPGES-1 inhibitors with IC_{50} values of 1.7-6.4 µM for mPGES-1 and 2.5-6.8 µM for 5-LO, respectively. Equal inhibition of both enzymes was achieved by the 4-chlorophenyl substituted lonazolac analogue **143** (IC_{50} =2.9 µM for 5-LO and IC_{50}= 2.3 µM for mPGES-1, respectively). Simultaneously, the structural modifications led to a loss of the inhibitory potency towards COX-1.

Thus, the development of dual inhibitors targeting LT and PG formation based on lead structures of known anti-inflammatory drugs offers the chance to a more specific and safe anti-inflammatory therapy.

7 Zusammenfassung

Leukotriene (LTs) stellen wichtige, von der mehrfach ungesättigten Fettsäure Arachidonsäure (AA) abstammende Lipidmediatoren mit einer umfassenden Beteiligung an pathophysiologischen Vorgängen dar. Das Enzym 5-Lipoxygenase (5-LO) katalysiert die ersten beiden Schritte der LT-Bildung aus AA, die aus Membranphospholipiden durch $cPLA_2$ freigesetzt wird. Im ersten Schritt wird dabei molekularer Sauerstoff auf AA übertragen, wobei 5-H(P)ETE entsteht, welches anschließend zum Epoxid-Zwischenprodukt LTA_4 dehydriert wird. Die weitere Umwandlung von LTA_4 durch die Enzyme LTA4H und LTC4S führt zur Bildung von LTB_4 sowie Cysteinyl-LTs. LTs werden mit vielen Erkrankungen wie Asthma, Atherosklerose, Osteoporose, kardiovaskulären Erkrankungen sowie verschiedenen Krebsarten in Zusammenhang gebracht. Dementsprechend stellt ein gezielter pharmakologischer Eingriff in die LT-Synthese einen vielversprechenden Ansatz zur Behandlung dieser Erkrankungen dar.

Die komplexe Regulation der katalytischen 5-LO-Aktivität ermöglicht verschiedenartige Angriffspunkte für die Hemmung der LT-Bildung. Die zelluläre Aktivität des Enzyms wird durch mehrere Faktoren wie Ca^{2+}, MAPK, Phospholipide (z.B. PC), Glyceride und CLP gesteuert. Desweiteren hängt die Aktivierung der 5-LO von der Konzentration an Lipidhydroperoxiden (LOOH) ab, die zur Oxidation des Eisenions im aktiven Zentrum notwendig sind.

Dementsprechend sind mehrere pharmakologische Vorgehensweisen zum Eingriff in die LT-Bildung durch 5-LO möglich. Neben einer direkten Hemmung der 5-LO sind ein Antagonismus an den LT bzw. CysLT-Rezeptoren, die Hemmung von FLAP oder anderen Enzymen des AA-Wegs wie $cPLA_2$, LTA4H, oder LTC4S sowie ein Eingriff in andere Schlüsselschritte der LT-Bildung denkbar. Beispielsweise hemmen FLAP-Inhibitoren die Übertragung der Arachidonsäure und verringern dadurch die Verfügbarkeit als Substrat für 5-LO. Die direkten 5-LO-Inhibitoren lassen sich in drei Arten unterteilen: (I)

Inhibitoren vom Redoxtyp reduzieren das Eisenion im aktiven Zentrum und entkoppeln dadurch den katalytischen Zyklus, (II) Eisenligand-Inhibitoren, welche das Eisenatom chelatieren und (III) Inhibitoren vom Non-Redox-Typ, die mit AA oder LOOH am Enzym konkurrieren. Desweiteren wurde eine vierte Art von Verbindungen mit einer andersartigen oder bis dato unbekannten Wirkungsweise definiert [202]. Allerdings scheiterten die meisten potentiellen Wirkstoffkandidaten aufgrund schwerer Nebenwirkungen wie Methämoglobinbildung oder anderer toxischer Effekte, unspezifischer Wirkung oder waren *in vivo* nicht wirksam. Bis heute sind nur der Eisenligandinhibitor Zileuton sowie Cys-LT-Rezeptorantagonisten zur Behandlung von Astma und allergischen Erkrankungen auf dem Markt. Dementsprechend bedarf es alternativer Konzepte und neuer Leitstrukturen für die Behandlung von LT-assozierten Erkrankungen.

In dieser Arbeit wurden neuartige Klassen von Hemmstoffen der Leukotrienbiosynthese identifiziert. Da die Wirksamkeit einer Testsubstanz stark von den Versuchsbedingungen abhängt, wurden ausführliche Untersuchungen durchgeführt, um den Mechanismus der 5-LO-Hemmung aufzuklären.

Ausgehend von früheren Untersuchungen von Landwehr et al. [236] wurde innerhalb einer Serie von Indol-3-carboxylaten die Verbindung **1a** (methyl 2-(3-chlorophenylamino)-5-hydroxy-1H-indole-3-carboxylate) als potenter Hemmstoff der 5-LO in intakten PMNL (IC_{50}= 2.4 µM) als auch im zellfreien Testsystem mit rekombinanter 5-LO (IC_{50}= 0.3 µM) identifiziert. Strukturelle Veränderungen des Indol-3-carboxylat-Gerüstes brachte eine Reihe von Benzo[g]indol-3-carboxylaten mit wesentlich verbesserter Wirksamkeit hervor. Verbindung **8a** (ethyl 2-(3-chlorobenzyl)-5-hydroxy-1H-benzo[g]indole-3-carboxylate) stellte sich in nachfolgenden Untersuchungen als direkter, nicht-kompetitiver und reversibler Hemmstoff der 5-LO (IC_{50} = 0.23 µM in intakten PMNL und IC_{50}= 0.03-0.1 µM im zellfreien System) heraus. Desweiteren verringerte Substanz **8a** die 5-LO-Produktbildung im humanen Vollblutassay

und zeigte *in vivo* bei der Carrageen-induzierten Brustfellentzündung in Ratten antiinflammatorische Wirksamkeit mit einer vergleichbaren Wirkstärke wie der bekannte Eisenligand-Inhibitor Zileuton. In weiteren Untersuchungen von A. Koeberle in unserem Arbeitskreis wurden die Benzo[g]indolcarboxylate als potente duale Inhibitoren von 5-LO und mPGES-1 ohne nennenswerten Einfluss auf COX-Enzyme charakterisiert. In diesem Zusammenhang war die 2-chlorophenyl-substituierte Verbindung **8b** (IC_{50}= 0.1 µM für mPGES-1 und 1.2 µM für 5-LO) die wirksamste Substanz unter den Benzo[g]indolcarboxylaten [230].

Untersuchungen zur Struktur-Wirkungsbeziehung von 1,4-Benzochinonen und entsprechenden polyphenolischen Verbindungen ergaben, dass die Art des Substituenten am 1,4-Benzochinon- bzw. polyphenolischen Gerüst die insgesamte Wirkstärke der Substanz bestimmt. Insbesondere voluminöse Gruppen wie Naphthyl- oder Dibenzofuranylsubstituenten begünstigen die Hemmung der 5-LO-Produktbildung. Erstaunlicherweise hemmten einige 1,4-Benzochinonverbindungen rekombinante 5-LO direkt, was die Bioaktivierung zur 1,4-Diphenolstruktur ausschließt und auf einen anderen inhibitorischen Mechanismus als Redoxaktivität hinweist. Da weder 12- noch 15-LO-Produktbildung durch ausgewählte Verbindungen signifikant beeinflusst wurde, kann eine Wechselwirkung mit dem gemeinsamen Redoxzyklus der Säugetier-LOs ausgeschlossen werden. Interessanterweise korrelierten die Wirkstärken *in vitro* mit den Ergebnissen aus Docking-Untersuchungen, in denen für die 1,4-Benzochinonverbindungen stabilere Wechselwirkungen mit 5-LO gezeigt wurden als für die polyphenolischen Substanzen [249]. Die 1,4-Benzochinonverbindung **16d** wies eine hohe inhibitorische Wirkung in PMNL auf (IC_{50}= 0.58 µM), aber war in Homogenaten von Neutrophilen und rekombinanter 5-LO (IC_{50}= 11 µM) weniger aktiv. Die Substanz **16d** zeigte selbst keine Radikalfängereigenschaften, aber anscheinend sind die reduzierenden intrazellulären Bedingungen für die inhibitorische Wirkung notwendig. $cPLA_2$, COX-1 und mPGES-1 wurden nicht beeinflusst, wohingegen **16d** die COX-2 hemmte, wenn auch mit niedrigerer Potenz (IC_{50} = 7.3 µM) als

5-LO [248]. Im humanen Vollblutassay, welches den *in vivo*-Bedingungen ähnelt, waren **16d** sowie die polyphenolische Catecholverbindung **18b** aktiv (IC_{50}= 9.1 µM bzw. 8-9 µM) und die weitere antiinflammatorische Wirksamkeit von **16d** *in vivo* wurde in zwei etablierten Tierentzündungsmodellen gezeigt [248].

Im Anschluss an Untersuchungen der Struktur-Wirkungsbeziehungen von Pyrazolpropansäurederivaten wurden 9 Substanzen, die verschiedenartig an C-5 und N-1 des Pyrazolgerüsts substituiert sind, für Untersuchungen zum Wirkmechanismus ausgewählt. Generell war die Hemmwirkung der Substanzen in intakten Zellen höher als in zellfreien Systemen wie PMNL-Homogenaten oder rekombinanter 5-LO. 2 Substanzen (**33, 48**) zeigten eine stärkere Hemmwirkung in Homogenaten von E.coli mit rekombinanter 5-LO beobachtet, wohingegen aufgereinigte rekombinante 5-LO nicht beeinflusst wurde, was einen Einfluss zellulärer Komponenten auf die Wirkung nahelegt. Bei variierenden Substratkonzentrationen sowie unterschiedlicher Aktivierung der 5-LO durch Calcium oder osmotischen Zellstress wurden uneinheitliche mechanistische Eigenschaften der Verbindungen beobachtet. Interessanterweise verminderten einige Phenyl- und Chinolin-substituierte Verbindungen (**44, 46, 48-50**) potent die COX-1-vermittelte 12-HHT-Bildung in Thrombozyten (IC_{50}= 1.8 µM für **44**, 0.3 µM für **46** and 0.014-1 µM für **48-50**) ohne Einfluss auf COX-2 oder mPGES-1. Diese duale 5-LO/COX-1-Hemmung erweitert damit die antiinflammatorische Wirkung der Substanzen. Der bei einigen Verbindungen beobachteten Einfluss auf die subzelluläre Verteilung der 5-LO erforderte *in vitro* hohe Substanzkonzentrationen und das Ausmaß war nicht vergleichbar mit der Positivkontrolle Hyperforin. Nach der Stimulation mit fMLP verursachten die Substanzen **44** und **46** eine starke Hochregulation der p38 MAPK-Phosphorylierung in PMNL, allerdings erklärt dieser Effekt die Hemmwirkung nicht. Insgesamt sind die Pyrazolpropansäurederivate als Hemmstoffe der zellulären 5-LO-Produktbildung anzusehen, wobei sie auch teilweise die COX-1-vermittelte PG-Bildung hemmen.

Die Anwendung eines kombinierten Liganden- und Strukturbasierten Pharmakophormodells brachte eine Gruppe von 8 strukturell unterschiedlicher Verbindungen hervor (**Table 19**). Die 4,5-Diarylisoxazolverbindung **78** hemmte die LT-Bildung in A23187-aktivierten PMNL (IC_{50}= 4.4 µM) sowie in aufgereinigter rekombinanter 5-LO bei 30 µM bis auf 40%. Die Zugabe von 40 µM exogener AA verringerte die Hemmwirkung in PMNL (IC_{50}= 6 µM). Durch die Einführung von Fluor am äußeren Phenylring und der Austausch einer Methylgruppe durch eine Carboxylfunktion konnte die Potenz um das dreifache im zellbasierten Assay und um das fast fünffache in rekombinanter 5-LO gesteigert werden.

Im Gegensatz dazu hemmte die Benzimidazolverbindung **83** potent die A23187-induzierte LT-Bildung (IC_{50}= 0.31 µM), jedoch war unter zellfreien Bedingungen kaum eine Hemmwirkung zu beobachten. Da mechanistische Eigenschaften von Nonredoxtyp-Inhibitoren und Radikalfängereigenschaften ausgeschlossen werden konnten, wurde FLAP als potentielles Target von **83** untersucht. Tatsächlich wurde eine Wechselwirkung von **83** und ähnlichen Verbindungen mit FLAP mit Hilfe von Dockingstudien und Protein-Fishing-Experimenten mit immobilisierter Substanz nachgewiesen [257], [273]. Desweiteren machte **83** teilweise die 5-LO-Translokation rückgängig, ein Fakt, der für andere FLAP-Inhibitoren bereits beschrieben wurde. Nachfolgende Strukturvariationen und Untersuchungen zu Struktur-Wirkungsbeziehungen, brachten weitere potente Analoga hervor, die das Benzimidazolgerüst als neuer Chemotyp für die weitere Entwicklung von anti-LT-Arzneistoffen nahelegen.

Die gleichzeitige Hemmung der LT- und PG-Biosynthese soll zu einer besseren gastrointestinalen Verträglichkeit und antiinflammatorischer Wirksamkeit führen. Deshalb verheißen duale 5-LO/mPGES-1-Hemmstoffe eine gute Alternative zu NSAIDs zu sein. Die strukturelle Veränderung bekannter NSAIDs durch die Einführung einer Sulfonamidgruppe verbunden mit einem lipophilen aromatischen Substituenten führte zu potenten dualen 5-LO/mPGES-1 Inhibitoren mit IC_{50}-Werten von 1.7-6.4 µM für mPGES-1 und 2.5-6.8 µM for

5-LO. Eine gleichmäßige Hemmung beider Enzyme wurde durch die 4-chlorophenylsubstituierte Verbindung **143** (IC_{50} =2.9 µM für 5-LO and IC_{50} = 2.3 µM für mPGES-1) erreicht. Gleichzeitig führten die strukturellen Veränderungen zu einem Verlust der Hemmwirkung auf COX-1. Folglich bietet die Entwicklung dualer Hemmstoffe der LT- und PG-Bildung, basierend auf Leitstrukturen bekannter antientzündlicher Arzneistoffe die Möglichkeit einer spezifischeren und sicheren antientzündlichen Behandlung.

8 References

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9 Contributions

(in order of appearance)

Fig. 18B: Experiments with compound 8a in whole blood (LPS/fMLP) were conducted together with Dr. C. Pergola at University of Tübingen

Table 7, Table 20: Carrageenan-induced-pleurisy experiments were made in the group of Prof. L. Sautebin, University of Naples, Italy

Table 8: mPGES-1 data was generated and published by A. Koeberle

Chapter 4.2.2.4: Molecular Docking experiments were performed in the group of. P. Reddanna at University of Hyderabad, India.

Table 24: All mPGES-1 Data was produced by Julia Seegers and Daniela Müller, University of Tübingen

Fig. 28: $cPLA_2$ experiments were made by Dr. Anja Schaible (University of Jena) (A) and Bianca Jazzar (University of Tübingen) (B)

Chapter 4.4.1 Development of the pharmacophore model, docking experiments and final selection of the test compounds were made by Dr. W. Altenhofen (Chemical Computing Group Cologne Germany) and the group of Prof. Banoglu (University of Ankara, Turkey),

10 Publications

10.1 Original publications

Karg EM, Luderer S, Pergola C, Bühring U, Rossi A, Northoff H, Sautebin L, Troschütz R, Werz O (2009) *"Structural optimization of 2-substituted 5-hydroxyindole-3-carboxylates as potent inhibitors of human 5-lipoxygenase"* ; J. Med Chem 2009, 52 (11), 3474–3483

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10.2 Poster presentations

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Luderer S. , Çalışkan, B., Eren G., Özkan Y., Banoglu E., Altenhofen W., Gerstmeier J. , Pergola C., Werz O. (2012) *"Identification of a benzimidazole-based leukotriene synthesis inhibitor by virtual screening targeting FLAP"* DPhG annual meeting 2012, Greifswald , Germany

10.3 Book contribution

coauthor for H.P.T. Ammon (editor), Hunnius Pharmaceutical Lexicon, 10th Edition, 2010

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12 Akademische Lehrer

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