On the assembly of the leukotriene biosynthetic complex in intact cells and its pharmacological inhibition

Dissertation

To Fulfill the
Requirements for the Degree of
„doctor rerum naturalium“ (Dr. rer. nat.)

Submitted to the Council of the Faculty
of Biology and Pharmacy
of the Friedrich Schiller University Jena

by Dipl.-Pharm. Jana Karin Gerstmeier

born on January 15th, 1984 in Mühlhausen

1st Reviewer: Prof. Dr. Oliver Werz; University of Jena

2nd Reviewer: Prof. Dr. Gerhard K. E. Scriba; University of Jena

3rd Reviewer: Prof. Dr. Sabine Grösch; University of Frankfurt

Date of submission: June 25th, 2015

Date of disputation: September 14th, 2015

TABLE OF CONTENTS

ABBREVIATIONS

aa	amino acids
AA	arachidonic acid
ATP	adenosine triphosphate
A23187	calcium-ionophore
C	carbon
Ca^{2+}	calcium ion
CLP	coactosin-like protein
COX	cyclooxygenase
cPLA$_2$-α	cytosolic phospholipase A$_2$-alpha
CRP	C-reactive protein
CVD	cardiovascular diseases
CYP 450	cytochrome p450
cys-LT	cysteinyl-leukotriene
DAG	diacylglycerol
DHT	dihydrotestosterone
DTT	dithiothreitol
EET	epoxyeicosatrienoic acid
ERK	extracellular signal-regulated kinase
Fe^{2+}	ferrous ion
Fe^{3+}	ferric ion
FLAP	5-lipoxygenase-activating protein
fMLP	N-formyl-methionyl-leucyl-phenylalanine
GFP	green fluorescent protein
GPx	glutathione peroxidase
GSH	glutathione
GST	glutathione S-transferase

HEK	human embryonic kidney
hERG	human ether-a-go-go gene
HETE	hydroxyeicosatetraenoic acid
HPETE	hydroperoxyeicosatetraenoic acid
HPLC	high performance liquid chromatography
HWB	human whole blood
IF	immunofluorescence
LO	lipoxygenase
LOOH	lipid hydroperoxide
LPS	lipopolysaccharide
LT	leukotriene
LX	lipoxins
MAPEG	membrane-associated proteins in eicosanoid and glutathione metabolism
MAPK	mitogen-activated protein kinase
MK	MAPK-activating protein kinase
MPO	myeloperoxidase
NLS	nuclear localization sequence
PBS	phosphate-buffered saline
PC	phosphatidylcholine
PG	prostaglandins
PKA	protein kinase A
PL	phospholipase
PLA	proximity ligation assay
PMA	phorbol 12-myristate 13-acetate
PMNL	polymorphonuclear leukocytes
ROS	reactive oxygen species
SRS-A	slow-reacting substance of anaphylaxis
TX	thromboxane

SUMMARY

Leukotrienes (LT) are potent lipid mediators derived from arachidonic acid (AA) via the 5-LO pathway [1]. First, AA is converted to 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HPETE), which in turn is metabolized to LTA_4. In the cell, 5-LO activity depends on a crucial translocation from the soluble compartment to the nuclear membrane-bound 5-lipoxygenase-activating protein (FLAP) to achieve endogenously released AA as substrate for LT biosynthesis [2]. Although 5-LO and LT biosynthesis is known for more than 35 years, a role in the development of cardiovascular diseases (CVD), cancer, and Alzheimer`s disease besides chronic allergic diseases like asthma has only recently been discovered [3]. Genetic knock-out of FLAP as well as pharmacological inhibition precludes metabolism of AA via the 5-LO/FLAP pathway upon stimulation [4].Therefore, FLAP displays a promising drug target for LT-associated diseases.

Inspired by the therapeutic potential, BRP-7 as a novel chemotype for FLAP inhibitors was identified in a virtual screening approach [5]. Unlike well-established FLAP antagonists, the core of BRP-7 is a non-acidic benzimidazole ring, lacking typical pharmacophoric moieties of well-established FLAP inhibitors. The first part of this thesis concentrates on the pharmacological characterization of BRP-7 *in vitro* and *in vivo* (manuscript I). The potent inhibition of BRP-7 in intact primary human monocytes (IC_{50}: 40 nM) and neutrophils (IC_{50}: 150 nM), as well as in human whole-blood (HWB) (IC_{50}: 4.8 µM) and two *in vivo* animal models of acute inflammation highlights the pharmacological potential of BRP-7 as FLAP antagonist. No significant inhibition of BRP-7 in cell-free assays or upon exogenous substrate supply to A23187-stimulated cells was observed, which is a common characteristic of FLAP inhibitors.

Detailed knowledge about the complex regulation of 5-LO and FLAP as key enzymes in LT biosynthesis is incomplete, and novel innovative molecular pharmacological approaches are rare. Moreover, FLAP as a membrane-embedded protein devoid of enzymatic activity hampers the study of FLAP outside the cellular context. So far it has been speculated in the literature that FLAP and 5-LO co-localize at the nuclear membrane upon stimulation to establish the biosynthetic LTA_4 complex. However, no distinct proof for a functional protein-protein interaction *in cellulo* has been given until

now due to insufficient experimental approaches. Consequently, putative FLAP inhibitors, and their influence on the LTA_4-biosynthetic 5-LO/FLAP complex could not be properly determined. Our laboratory was the first to establish a powerful cell-based model to overcome these difficulties. In brief, HEK293 cells were stably transfected with FLAP and/or 5-LO (manuscript II). Co-expression of 5-LO and FLAP significantly increased cellular 5-LO activity by supporting the biosynthesis of LTA_4 which was blocked by FLAP antagonists but not by direct 5-LO inhibitors. Moreover, 5-LO membrane association was strengthened in FLAP co-expressing cells. The results imply that FLAP not only positions AA into the active site of 5-LO but also the intermediate 5-HPETE to assure efficient LTA_4 formation. Thus, we identified a novel function of FLAP, that is, support of the LTA_4 synthase activity of 5-LO without marked effects on the 5-HETE production. Seemingly, FLAP acts as a scaffolding protein for the assembly of the LT-biosynthetic complex that occurs at the nuclear membrane of activated cells but whether 5-LO and FLAP physically interact remained elusive.

A novel and highly specific fluorescence technique, that is, the proximity ligation assay (PLA) [6], yields distinct proof for a direct *in situ* protein-protein interaction of native 5-LO and FLAP in HEK293 cells as well as primary human monocytes and neutrophils (manuscript III). For the first time, we identified a functional interaction between 5-LO and FLAP at a time point where 5-LO translocation, co-localization with FLAP and LT biosynthesis was already terminated. Pharmacological inhibition of FLAP as well as inhibition of AA release prevents an assembly of the 5-LO/FLAP complex without influencing 5-LO translocation and co-localization with FLAP. Exogenous supply of AA or 5-HPETE restored the interaction, supporting our hypothesis of FLAP being indispensable for the assembly of the LTA_4-biosynthetic complex by presenting AA and 5-HPETE as substrates to 5-LO.

Together, this thesis supports the role of FLAP for cellular LT biosynthesis, and provides novel insights related to FLAP as a rather regulating protein for 5-LO activity than simply an “activating” one. We speculate that FLAP controls the fate of AA metabolism towards LTA_4, and tempers the pro-inflammatory response by assembling a tight 5-LO/FLAP complex at the nuclear membrane to limit 5-LO activity. The results provide an excellent cellular model for the evaluation of putative FLAP antagonists on

one hand, and reveal significant insights into the functional 5-LO/FLAP interaction in primary human cells by PLA.

ZUSAMMENFASSUNG

Die enzymatische Umwandlung von Arachidonsäure (AA) über den 5-Lipoxygenase (5-LO)-Weg führt zu wichtigen Entzündungsmediatoren, den Leukotrienen (LT) [1]. Durch regiospezifische Wasserstoffabstraktion und anschließender Oxidation wird AA im ersten Schritt (Dioxygenaseaktivität) zum Peroxid 5(S)-Hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HPETE) metabolisiert, um dann in einem zweiten Schritt (Epoxidaseaktivität) in LTA_4 umgewandelt zu werden. In intakten Zellen ist für die zelluläre katalytische Aktivität der 5-LO eine Stimulus-induzierte Translokation aus dem Zytosol zu dem kernmembranständigen 5-Lipoxygenase-aktivierenden Protein (FLAP) essentiell [2]. Obwohl die Identität der LT sowie deren Biosynthese bereits vor 35 Jahren aufgeklärt wurde, weiß man erst seit kurzem, dass diese Lipidmediatoren neben Asthma und allergischer Rhinitis auch bei Alzheimer, Krebs und Herz-Kreislauf-Erkrankungen eine wichtige Rolle spielen. Sowohl genetischer Knock-out von FLAP in Mäusen als auch die pharmakologische Inhibition von FLAP als Target verhindert die Metabolisierung von AA zu pro-inflammatorischen LT über den 5-LO/FLAP Weg [4]. FLAP bietet somit vielversprechende Therapieansätze zur Behandlung LT-assoziierter Erkrankungen. Ein großer Vorteil der FLAP Inhibitoren ist die potente Wirksamkeit bei kardiovaskulären Erkrankungen, zusätzlich zu ihrem ausgeprägten Potential als Hemmstoffe akuter und chronischer Entzündungskrankheiten [7].

Auf der Suche nach innovativen und hochpotenten FLAP Antagonisten wurde im Rahmen dieser Arbeit erstmals ein kombiniertes Liganden- und Struktur-basiertes Pharmakophormodell für potentielle FLAP Inhibitoren entwickelt und BRP-7 als neue Leitstruktur für FLAP Antagonisten ermittelt [5]. Im Gegensatz zu etablierten FLAP Inhibitoren enthält BRP-7 einen Benzimidazolring als Grundstruktur, ohne die für FLAP Antagonisten charakteristische Säurefunktion. Der erste Teil dieser Arbeit beschäftigt sich mit der ausführlichen Charakterisierung der Pharmakologie von BRP-7 (Manuskript I). Die potente Inhibition der LT-Synthese durch BRP-7 in intakten primären humanen Monozyten (IC_{50}: 40 nM) und Neutrophilen (IC_{50}: 150 nM), sowie dessen starke Hemmung in humanem Vollblut (IC_{50}: 4.8 µM) als auch *in vivo* unterstreichen das hohe pharmakologische Potential von BRP-7. Wie erwartet fand

keine signifikante Hemmung der 5-LO Aktivität durch BRP-7 in zellfreien Untersuchungen oder nach Zugabe exogener AA zu intakten Zellen statt.

Das Wissen um die molekulare Regulation von 5-LO und FLAP als Schlüsselenzyme der LT-Synthese ist jedoch lückenhaft und innovative, molekularpharmakologische Ansätze sind stark limitiert. Hinzu kommt, dass FLAP als kernmembranständiges Protein keine enzymatische Aktivität besitzt. Bislang wurde in der Literatur spekuliert, dass FLAP und 5-LO an der Kernmembran ko-lokalisieren um dort in orchestrierter Weise miteinander zu interagieren. Nachgewiesen wurde diese zelluläre Interaktion aufgrund unzureichender experimenteller Methodik bislang nicht. Im Rahmen dieser Arbeit gelang es erstmals rekombinantes FLAP und/oder 5-LO stabil in HEK293 Zellen zu exprimieren (Manuskript II). Die Ko-expression von 5-LO und FLAP steigerte signifikant die 5-LO Produktbildung ausschließlich in intakten Zellen durch eine gezielte Erhöhung der LTA_4 Synthese, was durch FLAP Inhibitoren geblockt werden konnte. Weiterhin wurde die Membranassoziation der 5-LO nach Stimulation in FLAP exprimierenden Zellen deutlich gesteigert. Die Ergebnisse legen nahe, dass FLAP neben AA zusätzlich das Intermediat 5-HPETE am aktiven Zentrum der 5-LO positioniert. Dies stellt eine bisher unbekannte Funktion von FLAP dar.

Obwohl die Daten darauf hindeuten, blieb die Frage nach einer direkten Protein-Protein-Interaktion zwischen 5-LO und FLAP für den Substraterwerb und dessen Umsetzung zu den LT unbeantwortet. Mit der Etablierung einer hochempfindlichen und spezifischen fluoreszenzmikroskopischen Technik, dem Proximity Ligation Assay (PLA) [6], konnte erstmalig eine direkte *in situ* Protein-Protein-Interaktion zwischen 5-LO und FLAP in HEK293 Zellen sowie primären humanen Monozyten und Neutrophilen nachgewiesen werden (Manuskript III). Während Translokation und Ko-lokalisation mit FLAP parallel zur 5-LO Produktbildung verläuft, findet die 5-LO/FLAP Interaktion zeitverzögert statt. Sowohl die pharmakologische Inhibition von FLAP als auch die Hemmung der AA Freisetzung unterbindet eine Interaktion der beiden Proteine an der Kernmembran, ohne dass ein Einfluss auf das Translokationsverhalten der 5-LO durch die jeweiligen Inhibitoren beobachtet werden konnte. Interessanterweise konnte durch Zugabe exogener AA oder 5-HPETE die funktionelle 5-LO/FLAP Interaktion wieder hergestellt werden. Die Ergebnisse unterstützen die

Hypothese, dass FLAP unabdinglich für die LTA_4 Bildung ist, indem es das Intermediat 5-HPETE am aktiven Zentrum der 5-LO hält.

Zusammenfassend zeigen die neuen Erkenntnisse, dass FLAP nicht nur ein “aktivierendes”, sondern vielmehr ein regulierendes Protein für die 5-LO Aktivität darstellt. Anscheinend steuert FLAP die 5-LO Produktbildung und limitiert die pro-inflammatorische Antwort durch Etablierung eines inaktiven 5-LO/FLAP Komplexes an der Kernmembran was zu einer Beeindigung der Produktbildung führt. Die Ergebnisse tragen dazu bei FLAP Antagonisten zukünftig eindeutig anhand des HEK293 Zellmodels zu identifizieren und deren inhibitorisches Potential zur Ausbildung des LTA_4-synthetisierenden 5-LO/FLAP Komplexes im PLA an Primärzellen zu untersuchen.

1. INTRODUCTION

1.1 Eicosanoids in inflammation

Acute inflammation is a necessary and mainly beneficial host defense process that goes along with redness, pain, swelling and heat, accomplished by cells of the human immune system to eliminate the injurious stimuli, and induce healing [8]. Although host defense is mostly beneficial, hypersensitive responses to allergens contribute to excessive activation of immune cells, leading to uncontrolled manifestation of autoimmune diseases and chronic inflammation. Persistent and non-resolving inflammation forms the basis for a diverse field of pathophysiological human diseases including asthma, cancer and atherosclerosis [3, 9, 10]. Besides cytokines and factors of the complement system, lipid mediators derived from arachidonic acid (AA) capture an extraordinary role in this process as inflammatory signaling molecules. The first committed step in the formation of these inflammatory lipid mediators is the release of AA from phospholipids by cytosolic phospholipase A_2-α (cPLA_2-α). Dioxygenation of AA via two mayor pathways: the cyclooxygenase (COX) [11] and the lipoxygenase (LO) pathway [12] leads to a group of lipid mediators called eicosanoids, including prostaglandins (PG), thromboxanes (TX), lipoxins (LX), and leukotrienes (LT). Additionally, AA is converted by the actions of the cytochrome p450 (CYP 450) enzymes to the anti-inflammatory epoxyeicosatrienoic acids (EETs) (Fig. 1) [13]. So far, two mammalian COX isofomes (COX-1 and COX-2) and six mammalian LO isozymes are known in the human body. Among the six human LOs, classified by their regio- and stereoselectivity for AA oxygenation [14], 5-Lipoxygenase (5-LO) plays an extraordinary role during inflammation as catalytic enzyme in LT biosynthesis [15].

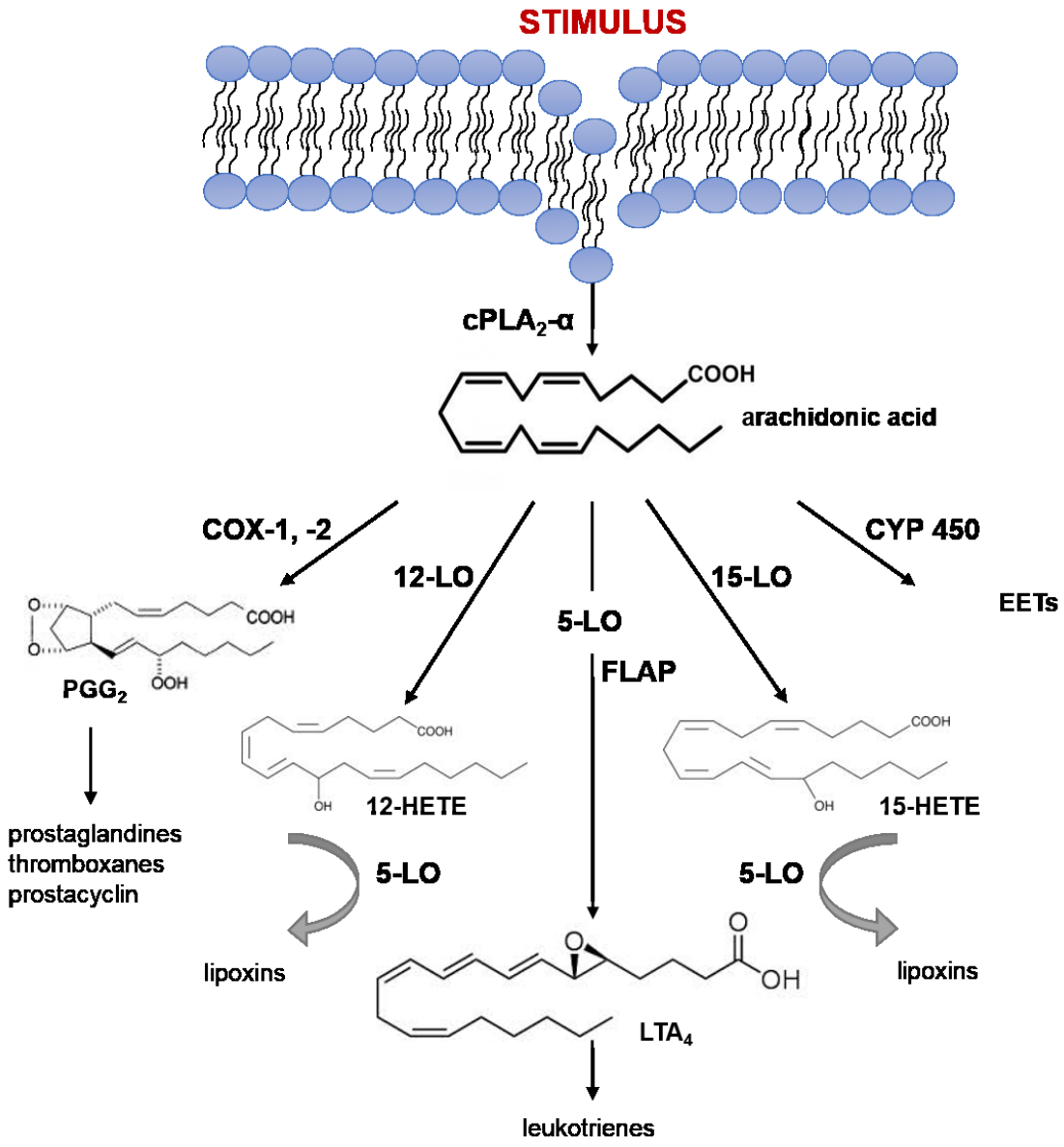


Fig. 1: Eicosanoid metabolism. Stimulus-induced AA release from phospholipids by cPLA$_2$-α is the first step in the eicosanoid cascade. AA is further metabolized by three different pathways. The COX pathway forms prostaglandins, thromboxanes and prostacyclin via the intermediate PGG$_2$. All LOs (e.g. 5-LO, 12-LO, 15-LO) synthesize H(p)ETE, depending on their regioselectivity to introduce oxygen. 5-H(p)ETE is further metabolized by a second 5-LO reaction to LTA$_4$, the common substrate for leukotrienes. LX production requires both, 15-LO (or 12-LO) and 5-LO activity. The CYP 450 pathway leads to the formation of EETs.

1.2 5-LO – key enzyme in leukotriene biosynthesis

1.2.1 Discovery of the leukotrienes

The term leukotriene is composed of “leuko”, attributed to their discovery in leukocytes, and “triene”, referring to three conjugated double bonds as common characteristics. More than 35 years of extensive studies have passed since their structure was first described. In 1976, Bengt Samuelsson, revealed the conversion of AA to 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HPETE) in rabbit polymorphonuclear leukocytes (PMNL) [16] that is rapidly reduced to the corresponding alcohol 5(S)-hydroxyeicosatetraenoic acid (5-HETE). Three years later, 5-H(p)ETE and LTB_4 were identified in human PMNL [17]. The latter is derived from LTA_4, an unstable epoxide that is formed from 5-HPETE by 5-LO [18]. Later, the "Slow-reacting substances of anaphylaxis" (SRS-A, described already in 1938) [19] were identified as a mixture of lipid mediators containing LTC_4, LTD_4 and LTE_4, collectively termed as cysteinyl-LTs (cys-LTs), formed by LTC_4 synthase (LTC_4-S) from LTA_4 [15].

1.2.2 Leukotrienes in human diseases

In general, LTs are known for their actions during acute inflammation and asthma bronchiale [3]. While LTB_4 is a potent chemokine that evokes chemotaxis and active movement of inflammatory cells upon stimulation, cys-LTs exhibit powerful bronchoconstrictive effects [20]. During the last three decades the list of LT-related diseases expanded, and chronic inflammation has been shown to be a risk factor for cardiovascular diseases (CVD) as well as for the development of cancer [9, 21]. Especially in the pathophysiology of atherosclerosis, a link between inflammation and the immune system is well accepted. Atherosclerotic plaques contain pro-inflammatory substances formed via the 5-LO pathway, and elevated levels of 5-LO products in atherosclerotic lesion promote plaque instability [21]. Moreover, increased expression levels of 5-LO, LTB_4 receptors and cys-LT1 receptor were observed in certain types of cancer [9], and 5-LO products released by cancer cells as well as invading cells contribute to survival and growth of the tumor [22]. On the other hand, transcellular

biosynthesis involving 5-LO and other LO activities provide mediators that contribute to resolution. The production of anti-inflammatory mediators like LXs or resolvins require for instances, the combined actions of 5-LO and 15-LO (or 12-LO) for successive AA oxygenation [23, 24].

1.2.3 Catalytic activity of 5-LO

5-LO, the key enzyme in LT biosynthesis, is a non-heme iron-containing dioxygenase that catalyzes the conversion of AA to LTA_4 in a two-step reaction [18]. The oxidation of the iron initiates the 5-LO catalytic cycle [25]. The inactive ferrous (Fe^{2+}) is oxidized to the active ferric (Fe^{3+}) form by lipid hydroperoxides (LOOH) [26]. Fe^{3+} is now coordinated by a hydroxide ion instead of water, enabling AA to enter the catalytic cavity [27]. The first AA-converting step is common for all other LOs. A hydrogen is stereospecifically abstracted to form a radical that undergoes a rearrangement for oxygen insertion. 5-LO abstracts the pro-S-hydrogen of carbon (C)-7 with a radical rearrangement to C-5, generating a trans double bond. Subsequently, oxygen is introduced at C-5 in an antarafacial manner to produce 5-HPETE. The intermediate 5-HPETE can either be non-enzymatically reduced by peroxidases to the corresponding alcohol 5-HETE or converted into the unstable epoxide LTA_4 by 5-LO in a second catalytic step. Therefor, the enzyme abstracts the pro-R hydrogen at C-10 with radical migration to C-6. The radical combines with the hydroperoxy group at C-5, followed by dehydration to yield LTA_4 [12, 28] (Fig. 2.). Among all LOs, this two-step concerted reaction is unique for 5-LO, and no other LO than 5-LO comprises a so-called LTA_4 synthase activity. The ratio of 5-H(p)ETE to LTA_4 depends on multiple regulating factors for 5-LO activity, assay conditions as well as cellular compartmentalization [29, 30]. 5-H(p)ETE production is favoured at high substrate and enzyme concentrations, for example if cells or purified 5-LO is stimulated with high concentrations of exogenous AA [31]. LTA_4 formation however, is prompted in intact cells by membrane binding [32] and association of 5-LO with its scaffolds coactosin-like protein (CLP) and 5-lipoxygenase-activating protein (FLAP) [2, 33]. Especially the induced nuclear membrane binding of 5-LO via its proposed membrane anchor FLAP promotes LTA_4 formation [34].

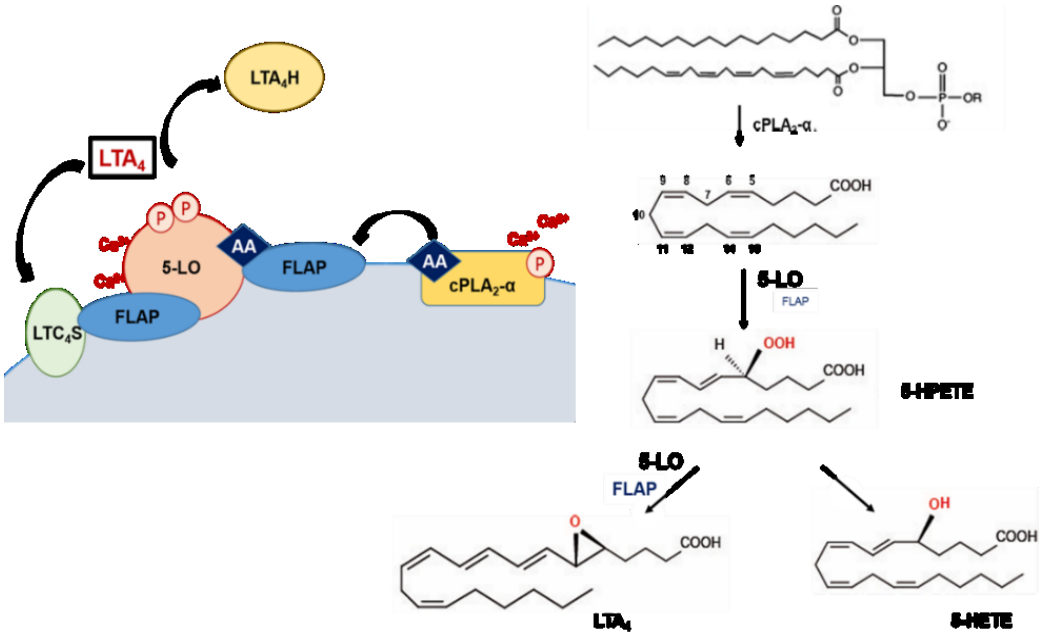


Fig. 2: 5-LO catalytic pathway. (Left) Schematic representation of cellular LT biosynthesis at the nuclear membrane upon stimulation. (Right) Metabolites and key enzymes of the 5-LO pathway.

1.2.4 Structure and function relationship

Purification and cDNA cloning of human 5-LO from various leukocytes revealed a soluble monomeric enzyme of 672 or 673 amino acids (aa) with a molecular weight of 72 to 80 kDa [35]. The first crystallized mammalian LO structure was the rabbit reticulocyte 15-LO in 1997 [36]. Many crystal structures of the LO family followed, but despite intensive attempts to crystallize human 5-LO for decades, researchers failed due to destabilizing protein sequences. The only available structure was a 15-LO crystal-based model, suggesting an N-terminal β-barrel regulatory domain and a C-terminal iron-containing catalytic domain for 5-LO. Finally in 2011, the first crystal structure of a so-called “stable-5-LO” was revealed (Fig. 3). Several mutations and deletions were applied to stabilize the protein for crystallization: replacement of three lysines ($Lys^{563-565}$) with Glu-Gln-Leu from coral 8(*R*)-LO, deletion of putative Ca^{2+}-

binding sites and membrane insertion residues (Δ40-44, Trp^{13}Glu, Phe^{14}His, Trp^{75}Gly, Leu^{76}Ser, Cys^{240}Ala, and Cys^{561}Ala) [27].

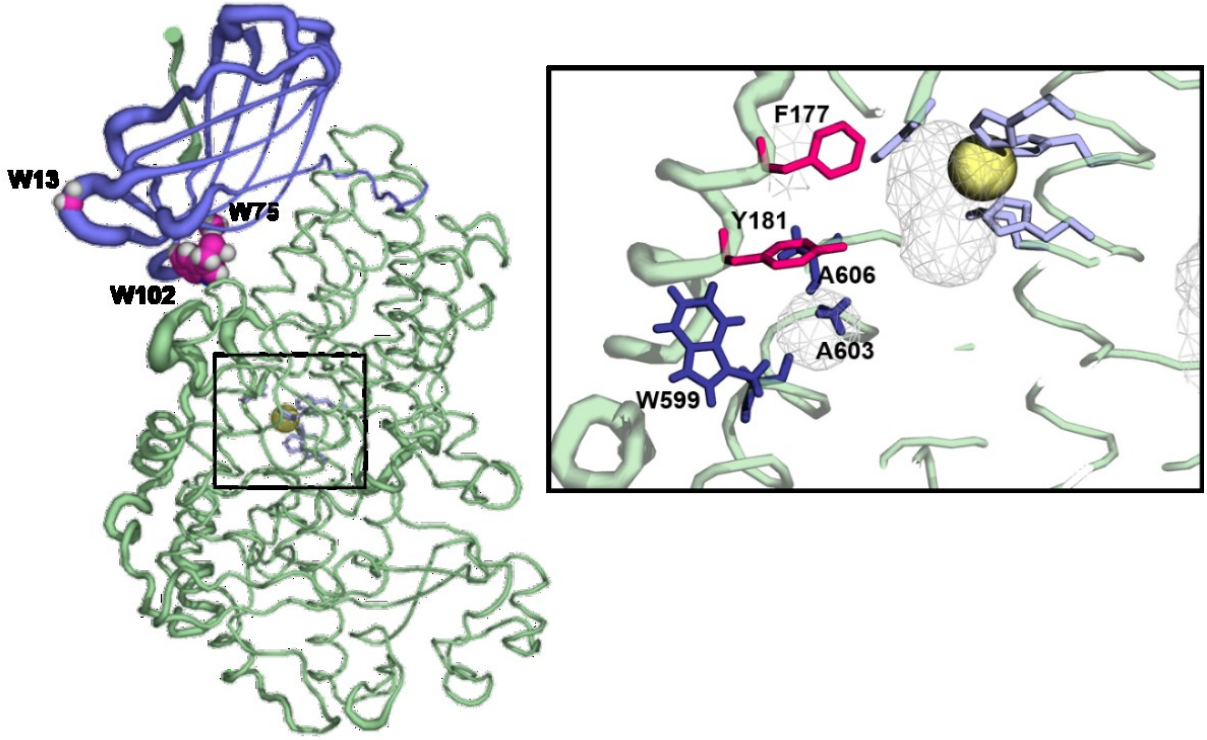


Fig. 3: Structure of "stable 5-LO". (Left) The cartoon rendering of the crystal structure of 5-LO (Protein Data Bank accession code, 3ORY) with the N-terminal C2-like domain (blue) and C-terminal catalytic domain (palegreen) containing the active site (lightblue) with the non-heme iron (yellow). (Right) Detail of the arched α-2 helix that closes the entry to the catalytic cavity. The potential AA entrance site is shown in stick rendering: Phe^{177} and Tyr^{181} (pink; "FY cork"), their respective counterparts Ala^{603} and Ala^{606} (blue) and stabilizingTrp^{599} (blue).

1.2.4.1 N-terminal regulatory C2-like domain

The smaller N-terminal C2-like domain (residues 1-120) is a mostly negatively charged β-barrel domain, containing several regulatory factor binding sites e.g. for calcium (Ca^{2+}), phosphatidyl choline (PC), and CLP [37-39]. Two four-stranded antiparallel β-sheets define the β-sandwich of the C2-like domain. Binding assays with radioactive-labeled Ca^{2+} suggest three important residues in loop 2 (Asn^{43}, Asp^{44}, and Glu^{46}) to be responsible for 5-LO activation via Ca^{2+} [37]. Ca^{2+}-binding to the C2-like domain is suggested to target 5-LO to the membranes [40]. It has been reported that 5-LO shows

high selectivity for PC and that this binding is Ca^{2+}-mediated by exposing three tryptophans (Trp^{13}, Trp^{75}, and Trp^{102}) to the surface [41]. Especially Trp^{75} has been shown to insert deep into the lipid bilayer. Since AA-esterified PC lipids are predominantly found in the nuclear membrane of cells [32], the Ca^{2+}-mediated preference for PC-binding is believed to initiate 5-LO translocation from the cytosol to the nuclear membrane. Indeed, a truncated GFP-tagged N-terminal domain construct, but not the respective C-terminal part, was sufficient for 5-LO translocation to the nuclear membrane [42]. This ability was solely observed for the N-terminal domain of 5-LO, and not for other LOs [42]. In line with these findings, human CLP was shown to bind and stabilize 5-LO *in vitro* via binding to the C-2 like domain. Especially Trp^{102} appears to be crucial for the association with CLP [43]. GST pull-down assays as well as yeast two-hybrid binding assays revealed human dicer as another interaction partner of 5-LO. Dicer has been described to bind to the N-terminal β-barrel domain, involving the three tryptophans mentioned above, and to increase 5-LO activity *in vitro* [44].

1.2.4.2 C-terminal catalytic domain

In contrast to the β-barrel sheets containing N-terminal domain, the C-terminal catalytic domain (residues 121-673) is mainly composed of α-helices, sheltering the non-heme catalytic iron in the center. An aspartate (Asn^{554}), three conserved histidines (His^{367}, His^{372}, and His^{550}) together with the C-terminal carboxyl end of Ile^{673} define the active site and coordinate the iron [27]. A water molecule resides at the sixth position. As described for many other non-heme iron containing enzymes, two histidines and one C-terminal carboxyl group are pivotal to anchor the iron. Mutational studies of 5-LOs active site suggest that His^{372}, His^{550}, and Ile^{673} form that triad, while His^{367} and Asn^{554} act as exchangeable ligands of the active site iron [45]. As described for soybean LO, an arched helix is covering access to the catalytic center [46], containing the highly conserved Leu^{414}. Together with Leu^{420} and Phe^{421}, this arched helix shields the catalytic site [27]. These residues have been described before for other LOs to be pivotal for substrate positioning and oxygen access.

The most unique part of the 5-LO structure is the configuration of the α-2 helix. While other LOs (e.g. human 15-LO-2 [47] and coral 8-LO [48]) contain a straight oriented six to seven turn long α-2 helix, the α-2 helix in 5-LO is a broken helix with three-turns, flanked by extended loops and a ~ 45° rotamer shift. Especially the position and orientation of the two bulky amino acids Phe^{177} and Tyr^{181} appear to close the entrance of AA to 5-LO`s active site, for which reason these two residues are often referred to as the "FY cork" [27]. Their bulky side chains are oriented towards the proposed substrate access channel with two alanines (Ala^{603} and Ala^{606}) across from them. The small side chains prompt the "FY cork" to point inwards and close the cavity, while Trp^{599} stabilizes the FY cork from the opposite side [27]. Eventually, product specificity among all AA-metabolizing enzymes is determined by two facts: (I) cavity depth, limiting how deep AA can slide into the cavity for attack, and (II) whether the substrate enters head (carboxyl) or tail (hydrocarbon) first. For 5-LO a head-first entry of AA is predicted [49]. A tail-first entry is limited by cavity depth (His^{600}) and would produce *R* stereochemistry instead of 5(*S*)-H(p)ETE. Taken together, the unique assembly around the "FY cork" defines the entrance of AA to the active site of 5-LO and controls regio- and stereoselective oxygenation of AA. While Phe^{177} is an invariant amino acid in other LO catalytic cavities, Tyr^{181} is specific for 5-LO. Instead of the bulky hydrophilic tyrosine, small hydrophobic aa reside at this position in other LO structures (Fig. 4). The "corked" cavity of 5-LO causes a lot of speculations about how AA can enter the active site.

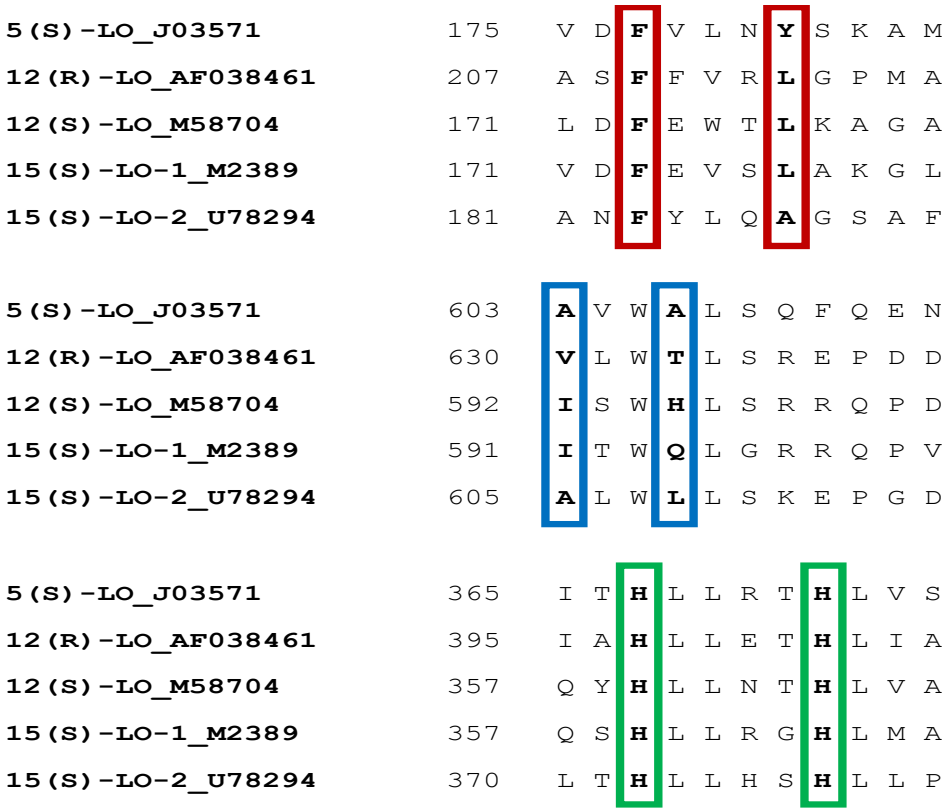


Fig. 4: Partial sequence alignment of 5(*S*)-LO and homologue position in other human LOs (Top) Residues defining the AA entrance site are surrounded in red (Phe^{177} and Tyr^{181} in 5-LO; “FY cork”). (Middle) Counterparts of the FY cork are surrounded in blue (Ala^{603} and Ala^{606} in 5-LO). (Bottom) Highly conserved histidines (His^{367} and His^{372} in 5-LO) coordinate the catalytic iron (green boxes).

1.2.5 Enzyme activity regulation

Among the AA-metabolizing pathways, the 5-LO pathway is probably the most complex regulated one. As described in detail below, 5-LO catalysis is strongly controlled by an accurate and fine-tuned machinery involving cofactors, second messengers, signaling kinase pathways, scaffolding proteins, redox status, compartmentalization, gender specificity, and self-inactivation [50].

1.2.5.1 Calcium, PC, and CLP

The second messenger Ca^{2+} was one of the first described modulating factors for LT biosynthesis [17]. Stimulation with Ca-ionophore (A23187) increases the permeability for Ca^{2+} ions, leading to an influx across the plasma membrane lipid bilayer into the cytosol. Half-maximal activity of purified 5-LO is obtained upon 1 - 2 µM, and full activity upon 4 – 10 µM Ca^{2+}, while lower concentrations (200 nM) are sufficient *in cellulo* [51]. Ca^{2+}-binding is needed for both catalytic activities of 5-LO [52]. Only slight 5-LO activity was demonstrated without Ca^{2+} addition under cell-free conditions [53]. Equilibrium dialysis together with gel filtration analysis revealed reversible binding of Ca^{2+} to 5-LO with a K_d of ~ 6 µM, and a stoichiometry of two Ca^{2+} per protein [54]. Recently, a third binding motif was suggested by the crystal structure of 5-LO [27]. Overall, enzyme activation via Ca^{2+} is characterized by a shorter lag phase, higher V_{max}, and decreased K_S for AA [53]. Ca^{2+}-binding modifies substrate accessibility as well as substrate turn-over. However, at high substrate concentrations (~ 40 µM AA), Ca^{2+} becomes redundant for purified 5-LO activity and crude cell homogenate preparations [55].

Ca^{2+} in general has been shown to bind to C2-like domains of various proteins, and mediates their membrane association. Indeed, mutational studies of the isolated His-tagged N-terminal C2-like domain of 5-LO verified specific binding sites for Ca^{2+} in loop 2 (Asn^{43}, Asp^{44}, and Glu^{46}) [37] (see 1.2.3.1). Ca^{2+} activation *in vitro* was improved by coincubation with cellular membrane fractions from leukocytes that were replaceable by lipid vesicles containing PC [52, 56]. Hence, Ca^{2+}-binding stimulates both 5-LO activity as well as membrane association, illustrating the complex regulatory mechanism of this second messenger. The positively charged ion is believed to neutralize the negatively charged aa of the N-terminal domain of 5-LO for an improved membrane association. In this context, three tryptophanes (Trp^{13}, Trp^{75}, Trp^{102}) were proposed to fulfill this function [41]. The preference of 5-LO for Ca^{2+}-induced PC-binding is believed to target cytosolic 5-LO to the AA-enriched nuclear membrane [38, 41]. Hence, the membrane association stabilizes both, 5-LO structure and activity. However, weak binding of 5-LO to PC vesicles *in vitro* was also shown in the absence of Ca^{2+} but without enzymatic 5-LO activity, implying that membrane binding alone is not sufficient for LT biosynthesis [38].

Besides cell membranes, the 16 kDa protein CLP was shown to function as a scaffold for 5-LO activity *in vitro* and *in vivo* [33]. It belongs to the actin-depolymerizing factor/cofilin family, resembling *Dictyostelium discoideum* coactosin that is known to bind F-actin. In a yeast two-hybrid screening the protein was identified as novel 5-LO interaction partner [57]. The binding stoichiometry of CLP to 5-LO is 1:1, while the binding of CLP to F-actin is 1:2. NMR structure analysis combined with site-directed mutagenesis revealed different residues for the binding of CLP to 5-LO (Lys^{131}) and F-actin (Lys^{75}). Although both residues are exposed on the surface in proximity to each other, no ternary complex has been observed so far. Instead, a competitive binding pattern of 5-LO and F-actin to CLP was demonstrated [58]. Co-immunoprecipitation experiments using epitope-tagged 5-LO and CLP in transiently transfected human embryonic kidney (HEK)293 cells confirmed CLP as 5-LO-binding protein in intact cells, supporting its relevance *in vivo* [43]. 5-LO was shown to migrate to the nuclear membrane from the cytosol in complex with CLP upon cell activation [33, 59], implying that the 5-LO/CLP association is permanent. The 5-LO/CLP association was shown to stabilize 5-LO structure and activity likewise since CLP prevented 5-LO from non-turnover self-inactivation *in vitro* [39]. Although the binding of CLP and 5-LO is Ca^{2+}-independent, no 5-LO activity was observed *in vitro* without Ca^{2+} addition, despite the presence of CLP. Thus, a similar mechanism for the support of 5-LO activity is suggested for CLP as discussed above for PC-binding [38]. Interestingly, in the absence of PC vesicles CLP primarily improved 5-H(p)ETE formation, whereas co-incubations with PC and CLP increased LTA_4 formation 3- to 5-fold [33]. As mentioned above, three tryptophanes (Trp^{13}, Trp^{75}, Trp^{102}) in the C2-like domain of 5-LO are important for the association with PC [41]. Thus, their relevance regarding the 5-LO/CLP interaction was examined. While Trp^{75} is mainly important for membrane insertion and plays a central role in 5-LO membrane-association, Trp^{102} appears to be essential for the binding to CLP [39]. Site-directed mutagenesis of this residue to alanine prevented CLP-binding as well as CLP-mediated activity support and structure stabilization. Computer-based docking models however, suggest another binding mode for CLP to 5-LO since Trp^{102} is not exposed to the surface, and located deep in an interdomain cleft. Instead, Trp^{102} might interact with Arg^{165} of 5-LO which in turn binds to Lys^{131} in CLP via a hydrogen bond [39].

1.2.5.2 ATP stimulation

Among all LOs, 5-LO is the only one capable to bind ATP. The nucleotide has been shown to stimulate the catalytic activity of 5-LO *in vitro* [60]. ATP at 0.1 - 2 mM is sufficient to increase 5-LO activity 2- to 6-fold in a Ca^{2+}-dependent manner with a K_d of 31 µM [53]. Besides ATP, other adenosine nucleotides (ATP > ADP > AMP) show similar stimulatory effects without ATP hydrolysis being involved [60, 61]. 5-LO binds to ATP-sepharose which is a routinely used purification method for the protein [62]. The ATP analog 2-azido-ATP covalently modified 5-LO at Trp^{75} and Trp^{201}, suggesting a role of the two aa or in proximity located residues in ATP-binding [62]. While Trp^{75} is located in the N-terminal domain, Trp^{201} resides in the catalytic domain. Stoichiometry analysis however, propose a 1:1 complex of 5-LO and ATP with homodimers of 5-LO in a head to tail confirmation to bring the two proposed ATP-binding motifs in proximity [63]. Subsequent site-directed mutagenesis of these residues however, did not influence 5-LO-binding to ATP-sepharose [64], and a truncated construct, lacking the N-terminal domain of 5-LO harbouring Trp^{75}, could still be stimulated by ATP [65]. Recently, indirubins were found to interfere with ATP binding to 5-LO, and these compounds effectively inhibited 5-LO product synthesis in an ATP-competitive manner [66].

1.2.5.3 Lipid hydroperoxides

The initial step of 5-LO catalysis is the oxidation of Fe^{2+} into Fe^{3+}, yielding the active form of the enzyme, accomplished by LOOHs [67]. 5-LO catalysis shows a characteristic lag phase after the addition of AA to purified 5-LO or crude cell lysates. The addition of various hydroperoxides like 5-HPETE, 12-HPETE, 15-HPETE or 13-HPODE reduce this lag phase [68]. Conditions promoting lipid peroxidation e.g. the formation of reactive oxygen species (ROS) by phorbol 12-myristate 13-acetate (PMA), depletion of glutathione (GSH), addition of peroxides (H_2O_2) or inhibitors of glutathione peroxidase (GPx) enzymes are beneficial for 5-LO activity [69]. Besides converting the iron into its active oxidized form, an increased peroxide tone upon oxidative stress might also activate 5-LO via phosphorylation, since oxidative stress was shown to induce the activation of p38 mitogen-activated protein kinase (MAPK) [61].

1.2.5.4 Phosphorylation

Phosphorylation plays a pivotal role in cell activation and turns protein functions on and off by altering their enzymatic activity and regulatory properties. Two key enzymes of the LT pathway 5-LO and $cPLA_2$-α are activated via phosphorylation, which significantly impacts their cellular activity and subcellular compartmentalization (see also 1.4) [70]. Extracellular signals induce phosphorylation that are able to elevate intracellular Ca^{2+} levels [71, 72].Thus, both mechanism are coordinated for maximal cell response during inflammation. Several phosphorylation motifs have been identified for 5-LO. In detail, three serine residues are phosphorylated by different protein kinases (Fig. 5). Ser^{271} is the target of p38 MAPK-activated protein kinase (MAPKAPK) 2/3, Ser^{663} of extracellular signal-regulated kinase 2 (ERK2), and Ser^{523} is activated by protein kinase A (PKA) [30]. First evidence for phosphorylated 5-LO was found in A23187-stimulated HL-60 cells, indicating that 5-LO activity and translocation is influenced by phosphorylation [73]. In brief, activated p38 MAPK phosphorylates and activates MAPKAPK 2/3 which phosphorylates 5-LO [73]. This can be blocked by the p38 MAPK inhibitor SB203580 [74]. In-gel migration assays combined with site-directed mutagenesis identified Ser^{271} as p38 MAPK-regulated phosphorylation site of 5-LO *in vitro* [72]. Interestingly, cell stress-induced 5-LO activation is Ca^{2+}-independent and offers opportunities to control 5-LO activity by phosphorylation [61] that is enhanced by polyunsaturated fatty acids like AA [74].

MAP kinase ERK2, was shown to phosphorylate Ser^{663} *in vitro* which can be prevented by the specific ERK activation inhibitor U0126 [75]. Although the stimulatory effect of ERK-mediated 5-LO phosphorylation is poor, exogenous substrate supply promotes 5-LO phosphorylation in a concentration-dependent manner as seen for p38 MAPK. It appears that dual phosphorylation at Ser^{271} and Ser^{663} occurs coordinated to enhance cellular 5-LO activity [75]. Since saturated fatty acids failed to support 5-LO activity *in vivo*, phosphorylation might only take place if free AA is available upon activation [75]. Like p38 MAPK-induced 5-LO phosphorylation, ERK2 activity was sufficient to increase 5-LO product formation and induce translocation in PMA-primed cells in the absence of Ca^{2+} mobilizing agents [71]. Of interest, p38 MAPK-regulated MK 2/3 or ERK2-mediated phosphorylation did not influence the activity of purified 5-LO,

suggesting a rather modulating role for the phosphorylation for 5-LO translocation and interactions *in cellulo* [76].

The third phosphorylation site of 5-LO is Ser^{523}, which is phosphorylated by the cAMP-dependent PKA [77]. Specific 5-LO phosphorylation by PKA activators or incubation with a phosphomimetic glutamate derivative reduced LT biosynthesis in cell-free preparations as well as transiently transfected mouse NIH-3T3 fibroblasts, and impaired 5-LO translocation in human neutrophils [77, 78]. Site-directed mutagenesis of Ser^{523} to alanine prevented 5-LO inhibition, and restored activity [77]. PKA-mediated phosphorylation of 5-LO was inhibited by exogenous supply of polyunsaturated fatty acids such as AA in neutrophils, leading to 5-LO activation and translocation to the nuclear membrane [79]. Note that Ser^{523} is located in proximity to the nuclear localization sequence (NLS) of 5-LO for which reason the phosphorylation of Ser^{523} reduced nuclear import of 5-LO, leading to an accumulation of 5-LO in the cytosol that was shown to reduce LT biosynthesis [80]. In this context, an allosteric regulation of phosphorylated Ser^{523} is thought to be involved in the control of 5-LO catalysis [77].

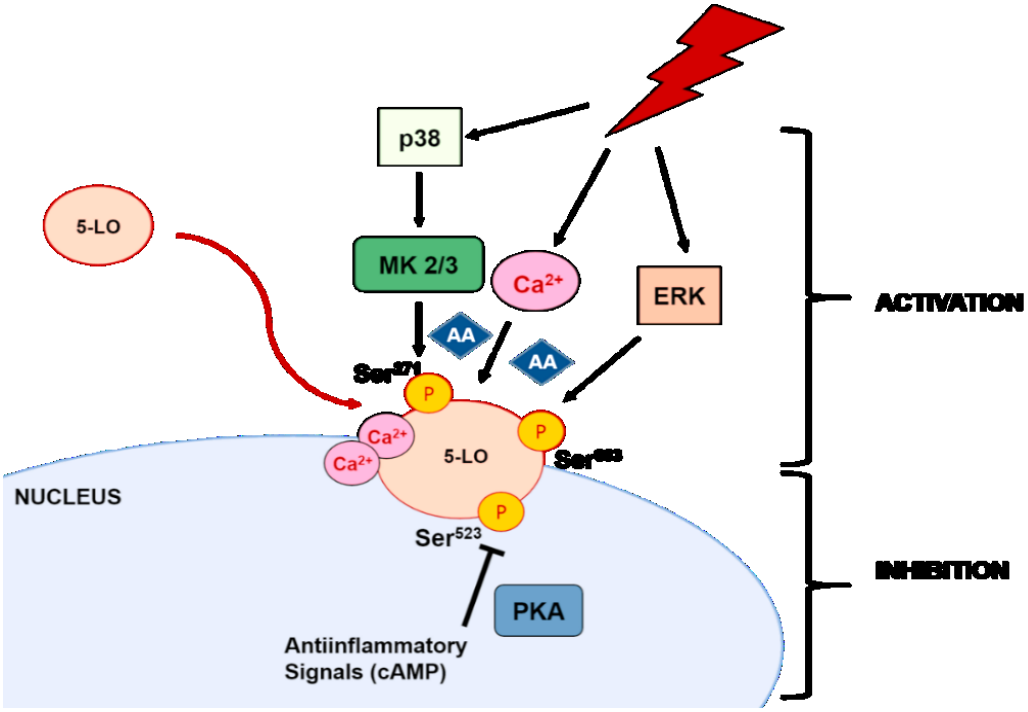


Fig. 5: Concerted cellular activation of 5-LO. Depending on stimulus and cell-type, 5-LO is regulated by Ca^{2+}-binding and/or phosphorylation. p38 MAPK-regulated MK 2/3 phosphorylates Ser^{271}, Ser^{663} is the target of ERK2, and Ser^{523} is phosphorylated by PKA.

1.2.5.5 Sex specificity

Besides their effects on sexual differentiation and reproduction, sex hormones have an impact on the human immune system. While estrogen is associated with pro-inflammatory events, testosterone seems to act anti-inflammatory [81]. Especially, LT-related diseases are more prominent in females. Recently, 5α-dihydrotestosterone (5α-DHT) was shown to up-regulate ERK2-mediated phosphorylation in male neutrophils that caused initial perinuclear localization of 5-LO in resting cells, which is believed to reduce 5-LO activity in male neutrophils upon stimulation [82]. Besides male neutrophils, reduced levels of 5-LO products were also observed in male monocytes and human whole blood [83]. In monocytes however, the translocation pattern of 5-LO was marginal affected by androgens. Here, ERK2 activation reduced phospholipase D (PLD) activity which could be restored by exogenous supply of diacylglycerol (DAG) [83]. The gender differences observed *in vitro* were confirmed *in vivo* in the zymosan-induced peritonitis model with male and female mice [84]. Furthermore, female mice showed higher mortality rates than male mice in a mouse model resembling human systemic lupus erythematosus (MRL-lpr/lpr), and the testosterone-mediated beneficial effect was abolished upon 5-LO gene knock-out [85]. The improved knowledge about gender bias-related differences in 5-LO activity may contribute to understand and explain the predisposition of females for LT-associated diseases.

1.2.5.6 5-LO self-inactivation

Two mechanism contribute to 5-LO self-inactivation, non-turnover-dependent structure instability (see also 1.2.4) and turnover-based suicide inactivation. Compared to other LOs, 5-LO is a fragile enzyme, a property that may be necessary to temper the pro-inflammatory response. Purified 5-LO, for example, has a half-life of 24 h at 2 °C [86]. Non-turnover-dependent 5-LO inactivation involves a reaction of the central iron with hydroperoxides, resulting in ROS [51]. In this context, reducing agents like dithiothreitol (DTT) or GSH were shown to stabilize 5-LO and prolong 5-LO activity [87]. In addition, CLP acts as a stabilizing scaffold for 5-LO structure and activity by preventing non-turnover based inactivation [39]. Another mechanism is 5-LO turnover-based suicide inactivation that occurs during 5-LO catalysis. If purified 5-LO is treated with Ca^{2+},

substrate, and PC vesicles in air-saturated buffer its half-life is reduced to ~ 30 sec. The rate of inactivation is predominantly dependent on substrate accessibility and concentration [88]. Both 5-LO products 5-HPETE and LTA_4 are highly reactive substances that were shown to inactivate the enzyme irretrievable [89, 90]. Despite the fact that 5-LO is a fragile enzyme due to turnover and non-turnover inactivation, pro-inflammatory LT biosynthesis still occurs, and nanomolar concentrations are sufficient for further metabolism by downstream enzymes.

1.3 5-Lipoxygenase-Activating Protein (FLAP)

1.3.1 Discovery of FLAP and inhibition

In 1989, scientists at Merck-Frosst discovered that the indole-class inhibitor MK886 potently suppresses *in cellulo* LT biosynthesis without affecting 5-LO or $cPLA_2$-α [91]. Scientists at Bayer identified a quinolone-type inhibitor (BAY X-1005) with similar activity [92]. Both compounds potently inhibit LT formation in intact cells after stimulation (e.g. with A23187) but lose their efficacy in cell homogenates or in studies with purified 5-LO. Dixon et al. cloned and identified the MK886-binding protein as a determinant for LT-biosynthesis in intact cells and termed it “Five-lipoxygenase-activating protein” or short FLAP [2]. FLAP is a 18 kDa nuclear membrane-embedded protein that is believed to transfer endogenously released AA to 5-LO for metabolism [4]. Further drug developments resulted in indole-quinolone hybrids as MK591 [93]. Site-directed mutagenesis of FLAP combined with MK591-radioligand binding studies determined the inhibitor binding site, and suggest a partially overlapping binding site with AA [94, 95]. For all three compounds phase I and phase II clinical data in the treatment of asthma are available. However, neither one reached phase III clinical studies, for unknown reasons [96-98]. Recently, scientists at GlaxoSmithKline presented a novel FLAP inhibitor (GSK2190915), similar in structure to the compounds mentioned above (see Fig. 6). GSK2190915 successfully completed phase II clinical studies and entered phase III with promising perspectives to become the first FLAP inhibitor on the market for the treatment of chronic asthma [99]. Besides their potency in chronic allergic and respiratory diseases, animal studies as well as genetic knock-

out experiments propose an emerging role for FLAP inhibitors in atherosclerosis, myocardial infarction, and stroke. MK886 for example was shown to prevent plaque formation and even stabilized those in a mouse atherosclerosis model [100, 101]. Additionally, variants of the FLAP gene (ALOX5AP) were shown to be a risk factor for heart attacks or stroke, and high levels of LTs are one of the determinants for the developing of CVD [102, 103]. To sum up, FLAP inhibitors were reported to decrease LTB_4, myeloperoxidase (MPO), and systemic C-reactive protein (CRP) levels [104]. Thus, the widespread application and therapeutic potential of FLAP inhibitors is enormous.

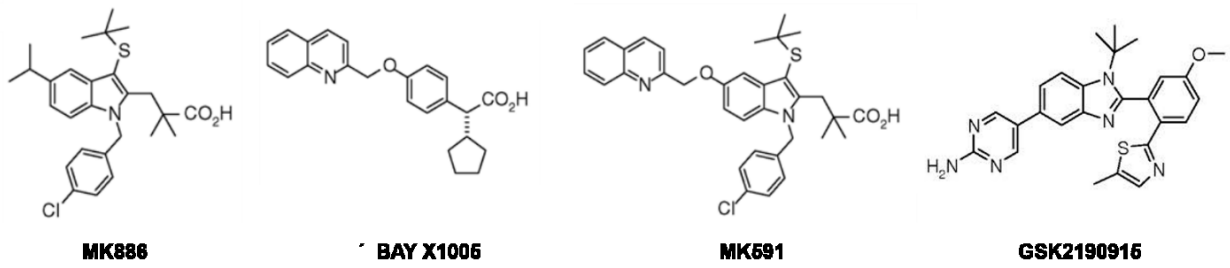


Fig. 6: Chemical structures of FLAP inhibitors. Despite high efficacy in early asthma trials (phase I and II), the first three compounds failed to enter the drug market but GSK2190915 (left) is a promising compound with high efficacy in phase III clinical studies.

1.3.2 Structural properties of FLAP

Due to sequence homology and structural properties, FLAP is a member of the membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) superfamily [105]. Within this family FLAP appears to resemble a subclass. The protein does not possess any enzymatic activity or a glutathione binding site, unlike the other MAPEGs that either conjugate GSH to their substrate or metabolize GSH during their catalytic cycle. Among all MAPEGs, FLAP and LTC_4S share the highest overall sequence identity with 31%. [105]. Both proteins form functional trimers with four transmembrane domains spanning the nuclear membrane, which are nearly superimposable [4]. In 2007, the three-dimensional structure of human FLAP was solved at 4.0 Å resolution in complex with MK591 [100]. Each FLAP monomer consists of four transmembrane spanning helices, connected by two cytosolic loops and one

lumenal loop. Both the N- and the C-termini are located in the perinuclear space. The flattened cytosolic top together with the pointed luminal base determine FLAP`s cylindric shape. The MK591-binding site is located deep in a groove on the membrane-embedded surface between helices of neighboring protomers (Fig. 7). In detail, MK591 forms van der Waals interactions with Val^{20}, Val^{21}, Gly^{24}, Phe^{25}, and Ala^{27} from helix α1; Tyr^{112} and Ile^{113} from cytosolic loop C2; Ala^{63} from helix α2; and Ile^{119}, Leu^{120}, and with Phe^{123} from helix α4 of the adjacent monomer. Three MK591 molecules bind to one FLAP trimer, resulting in a stoichiometry of 1:1. [100]. Sequence alignment of various FLAP species revealed highly conserved residues in two cytosolic loops, connecting helix α1 to α2, and helix α3 with α4. Thus, these surface loops might be promising candidates to interact with 5-LO. The deep inhibitor-binding groove is consistent with a hypothesis about lateral diffusion of AA molecules through the lipid bilayer, and might explain how FLAP inhibitors function as competitive inhibitors for AA-binding. Subsequent AA-binding to FLAP is believed to induce a conformational change of FLAP, especially of helix α2 [100]. The inhibitor binding stoichiometry suggests a similar stoichiometry for AA, resulting in a 1:1 complex of one FLAP trimer with one 5-LO monomer and three AA molecules. Interestingly, a similar hidden binding pocket for LTA_4 is described for the structurally-related LTC_4S. Thus, a selective lateral transfer of LTA_4 from FLAP to LTC_4S within the perinuclear region is also conceivable, and a complex of FLAP and LTC_4S is likely to be formed in order to produce LTC_4 [106]

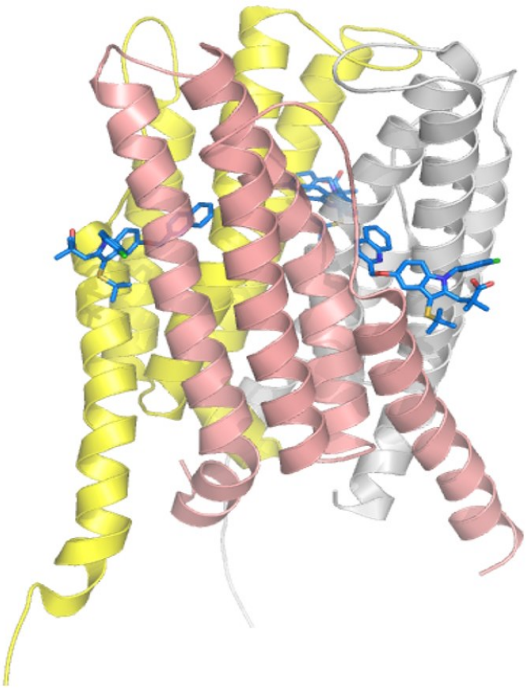


Fig. 7: Inhibitor-bound 5-Lipoxygenase-Activating Protein (FLAP). Cartoon representation of human trimeric FLAP with MK591 (Protein Data Bank accession code, 2Q7M). Monomers colored in yellow, grey, and rose. MK591 (blue sticks) is bound at membrane-embedded pockets of each monomer. Cytosolic (top) and nucleoplamic loops (bottom) may determine the 5-LO interaction site.

1.3.3 Role of FLAP in leukotriene biosynthesis

Among the mammalian LOs, 5-LO is unique for the strong dependency on its helper-protein FLAP in order to embrace full catalytic activity in intact cells. Intracellular Ca^{2+} release and subsequent phosphorylation redistributes 5-LO and $cPLA_2$-α to the membranes (see 1.4). The nuclear membrane-embedded protein FLAP presents AA to 5-LO, and aides the initial step in LT biosynthesis by favoring the dehydration of 5-HPETE to LTA_4 [34]. So far, efficient substrate transfer as well as 5-LO activity support are the two known functions of FLAP. After AA is released, it is supposed to be bound by FLAP and subsequently handed over to 5-LO [107]. Genetic knockout of FLAP as well as pharmacological FLAP inhibition abolishes 5-LO product formation after stimulation [108]. In fact, exogenous supply of AA partially circumvents the need for FLAP as transfer protein in LT biosynthesis. Same effects were observed in osteosarcoma cells transiently expressing 5-LO but lacking FLAP [109]. Although these cell lines failed to produce LTs from endogenous sources, substantial amounts of 5-LO products were formed upon exogenous substrate supply. Of interest, co-expression of FLAP in these cell lines improved the utilization of exogenous AA [2]. In consistence with these data, cell homogenates from human leukocytes or purified 5-LO completely depend on exogenous substrate supply, and FLAP inhibitors lose their efficacy under these assay conditions.

It is proposed that FLAP functions as a membrane anchor protein for 5-LO at the nuclear membrane after stimulation-induced translocation, and that FLAP inhibitors interfere with this translocation mechanism to prevent LT biosynthesis [110]. However, no convincing data for a protein-protein interaction of the native proteins in human leukocytes have been published so far. Although *in vitro* pull-down assays with artificial GST-tagged FLAP and 5-LO [111], or association studies using membrane-permanent cross-linking agents to perform fluorescence lifetime imaging microscopy (FLIM) in mouse neutrophils and rat RBL-2H3 cell lines [106] strongly suggest a direct interaction, the mode of association at the nuclear membrane is still vague. Moreover, pure co-localization studies with fluorescence microscopic techniques do not necessarily imply a physical protein-protein interaction.

1.4 Compartmentalization of LT biosynthesis

The biosynthetic formation of LTs in the cell is strongly compartmentalized with enzymes distributed in the cytosol, extracellular space as well as nuclear membrane. This regulatory intracellular compartmentalization controls localization, catalytic activity and protein-protein interactions of key enzymes of the LT synthetic cascade. Thus, intervention with the subcellular localization of the enzymes/proteins offers an opportunity to prevent excessive LT biosynthesis.

1.4.1 Subcellular localization of cPLA$_2$-alpha

The main substrate for 5-LO is AA, a polyunsaturated omega-6 fatty acid, predominantly found esterified in the nuclear membrane of cells [38]. The reacylation as well as the liberation rate limit the accessibility of free substrate for eicosanoid metabolism [112]. Among the known 15 PLA$_2$ proteins, cPLA$_2$-α is the only phospholipase that selectively cleaves AA in the sn-2 position of 1-palmitoyl-2-arachidonoyl-sn-glycerophosphocholine from the nuclear membrane lipid bilayer [113]. Similar to 5-LO, cPLA$_2$-α contains a N-terminal C2-like domain that binds Ca^{2+} and targets the enzyme to the AA-enriched perinuclear region, including the endoplasmic reticulum, Golgi apparatus, and nuclear envelope [114]. The C-terminal catalytic domain is distinct from other lipases and harbors an unusual Ser^{228}/Asp^{549} dyad to control specificity for AA. The structure suggests a flexible lid that undergoes a conformational change after membrane binding in order to gain substrate access [115]. Besides Ca^{2+}-binding, phosphorylation at Ser^{505} is sufficient to target the enzyme to the membrane, but maximal cPLA$_2$-α activation involves both, as seen for 5-LO activation [114, 116]. Consequently, 5-LO and cPLA$_2$-α act in a concerted manner upon cell activation and subcellular movement. The central role of cPLA$_2$-α in AA metabolism is impressively demonstrated in cPLA$_2$-α-deficient mice. Macrophages derived from these animals were not able to produce any eicosanoids and did not develop bronchial hyperreactivity in an anaphylaxis model upon stimulation [117].

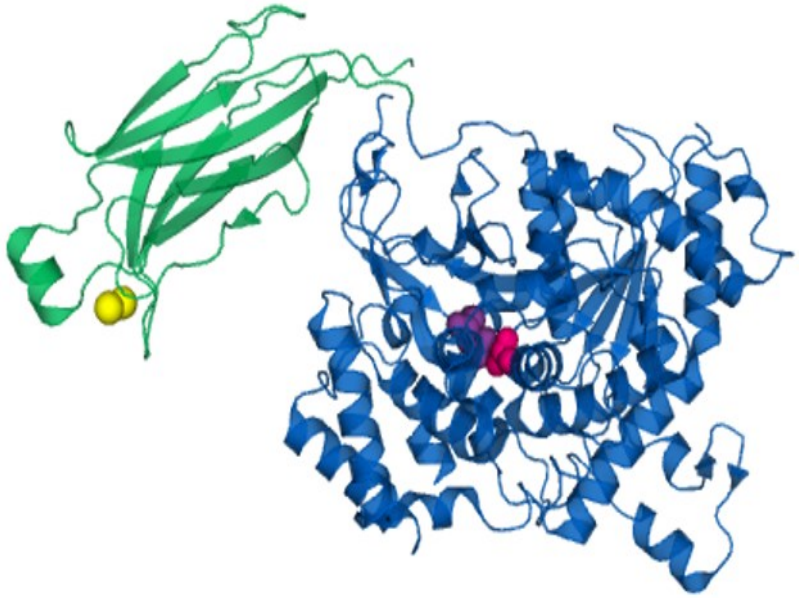


Fig. 8: Structure of cytosolic phospholipase A_2-alpha. cPLA2-α is rendered in cartoon (Protein Data Bank accession code, 1CJY). The N-terminal C2-like domain is colored palegreen with two bound Ca^{2+} ions (yellow spheres). The mainly α-helical C-terminal catalytic domain is colored in blue, harboring the catalytic dyad Ser^{228} (pink) and Asp^{549} (purple).

1.4.2 Subcellular localization of 5-LO

Subcellular localization determines the fate of 5-LO activity and product formation. In unstimulated cells, 5-LO resides in the cytosol or nucleoplasm, depending on the cell type and cellular environment. While 5-LO is mostly cytosolic in primary neutrophils, peritoneal macrophages, differentiated HL-60, and Mono Mac 6 cells, 5-LO is equally distributed between cytosol und nucleus in primary monocytes, alveolar macrophages, mouse bone marrow-derived mast cells, rat basophilic leukemia cells, Langerhans cells of human skin, and in mast cells [50]. Interestingly, in 5-LO transfected cell lines e.g. COS, CHO, NIH 3T3 or in cancer cell lines like RAW macrophages, 5-LO is predominantly intranuclear [20]. 5-LO nuclear import depends on the NLS which appears to be regulated by phosphorylation. Phospho-Ser^{271} supports the nuclear import while phospho-Ser^{523} seems repressive [118, 119]. Site-directed mutagenesis of the NLS or/and respective regulation of respective phosphorylation sites significantly decreased LTB_4 biosynthesis by 60–90%, which goes along with impaired intranuclear localization [80], and highlights the crucial role of 5-LO`s subcellular localization for activity.

There is evidence that intranuclear 5-LO translocates mostly to the inner nuclear membrane, whereas cytosolic 5-LO traffics to the endoplasmic reticulum and outer nuclear membrane. Thus, the different membrane preference seems to determine the

fate of 5-LO downstream products with higher LTB_4 levels of intranuclear 5-LO. The data are consistence with observations during adhesion and inflammation processes where 5-LO is directed from the cytosol to the intranuclear region of activated neutrophils [80]. On the other hand, targeting cytosolic 5-LO to the outer nuclear membrane appears to promote the formation of cys-LTs by LTC_4S [106]. In contrast to LTC_4S, FLAP is expressed in both, the inner and outer nuclear membrane. Two pools of FLAP have been identified: non-associated FLAP at both sides of the nuclear membrane, allowing nuclear soluble LTA_4H to produce LTB_4, and LTC_4S-associated FLAP at the outer nuclear membrane [106]. Different interaction sites of FLAP with 5-LO and LTC_4S are predicted without a direct protein-protein interaction of 5-LO and LTC_4S [106]. It is most likely that the FLAP/LTC_4S interaction is necessary to channel the final 5-LO product LTA_4 through the lipid bilayer from 5-LO to LTC_4S via FLAP.

Several studies suggest a multi-enzyme LT biosynthetic complex at the perinuclear region to achieve proper 5-LO activity and LT formation, involving $cPLA_2$-α, FLAP, 5-LO, CLP, and eventually LTC_4S and LTA_4H for further product metabolism [120]. FLIM analysis in mouse neutrophils already hypothesize a stimulation-dependent complex of 5-LO and FLAP, and a membrane association-mediated improved LTA_4 formation [120]. The subcellular movement of 5-LO to the nuclear membrane requires a variety of stimulating factors like Ca^{2+}-binding [121], and post-translational modifications like phosphorylation [71]. These activity regulating factors target 5-LO to the nuclear membrane, but FLAP is believed to anchor the protein at this locale [122]. Studies with FLAP inhibitors, competing for the AA-binding site, strongly suggest a regulating role of AA within this complex [94, 107]. Taken together, the knowledge about the complex mechanisms involved in substrate transfer and 5-LO/FLAP interaction is still vague.

1.4.3 Downstream enzymes of the 5-LO pathway

Depending on the expression of 5-LO downstream enzymes and 5-LO`s subcellular localization, 5-LO`s major product LTA_4 can be converted by two separate pathways to the respective pro-inflammatory mediators [50]. LTA_4 is either conjugated with reduced GSH by the nuclear membrane-embedded LTC_4S to form cys-LTs or is regio-

specific hydrolysed by soluble LTA_4H to produce LTB_4. These pro-inflammatory mediators can either be formed within one cell or by transcellular trafficking [123].

Like FLAP, LTC_4S is a nuclear membrane protein and belongs to the MAPEG superfamily. There is some evidence for a biosynthetic functional complex of FLAP and LTC_4S at the outer nuclear membrane [106]. Crystal structures are published for both proteins. The LTC_4S structure was resolved in complex with GSH and a detergent-like mimic of LTA_4 [124]. As seen for FLAP, the binding sites for GSH and LTA_4 lie deep in a groove of the membrane bilayer. The deep substrate positioning requires lateral diffusion through the membrane as discussed for FLAP. Note that GSH slides deeper into the lipid bilayer than LTA_4, and therefore is supposed to bind first and induce a conformational change for subsequent LTA_4-binding [124]. However, an opposite binding mode is also possible and has not been precluded yet. Attack of the anionic thiol group of GSH on C-6 of the LTA_4 epoxide ring yields the key compound of the cys-LTs, the LTC_4 [125, 126]. After metabolite export outside the cell successive cleavage of the glutamate and glycine residues lead to LTD_4, and eventually LTE_4. All three forming the group of cys-LTs. They act in nanomolar concentrations on G-protein coupled receptors, designated $cysLT_1$ and $cysLT_2$ [127]. While neutrophils lack LTC_4S, high enzyme levels are present in monocytes, macrophages, mast cells, platelets and eosinophils [126, 128].
An alternative fate for LTA_4 is the LTA_4H-mediated hydrolysis of the unstable epoxide ring to the chemoattractant mediator LTB_4. In 2001, the crystal structure of the bifunctional (hydrolase and aminopeptidase activity) zinc-containing LTA_4H was solved [129]. Human soluble LTA_4H is expressed in neutrophils, monocytes, macrophages, lymphocytes, eosinophils and erythrocytes, whereas platelets and basophils lack the enzyme [130]. Besides the hydrolase function for LTB_4 biosynthesis, the biological relevance of the aminopeptidase activity has not been fully determined yet. Recently, it was reported that the aminopeptidase activity cleaves the chemotactic tripeptide Pro-Gly-Pro, and inactivation of this peptide appears to be beneficial in the resolution of inflammation [131, 132].

Besides these two pivotal downstream pathways, with LTA_4 as the common substrate, the concerted catalytic actions during transcellular inflammatory response of 5-LO and 15-LO (or 12-LO) lead to anti-inflammatory mediators like LX [23, 133].

2. AIM OF THESIS

The overall aim of this thesis was to reveal the functionality and molecular basis for the assembly of the LT-biosynthetic 5-LO/FLAP complex at the nuclear membrane, and its pharmacological inhibition. The capacity of 5-LO to produce LTs from endogenous released AA is believed to be aided by FLAP [107]. Although FLAPs role in LT biosynthesis is known for more than three decades, the concerted action of FLAP with LT synthetic enzymes (e.g. 5-LO), and their complex assembly at the nuclear membrane are poorly understood. 5-LO product synthesis depends on a fine-tuned translocation process from a soluble compartment to the nuclear membrane-embedded helper-protein FLAP. The mechanism of action however, are incompletely solved so far. It is challenging to understand how FLAP facilitates 5-LO activity, and if a direct protein-protein interaction occurs within this process. Genetic knock-out of FLAP [108] or inhibition [110] completely abolishes LT biosynthesis *in vitro* and *in vivo*, turning FLAP into a promising drug target for the treatment of LT-associated diseases. In the light of these facts, inhibition of FLAP instead of 5-LO appears to be the more valuable strategy. FLAP however, is an integral membrane protein devoid of enzymatic activity [4], excluding evaluations in cell-free assays.

Specific objectives:

1) Characterize the *in vitro* and *in vivo* pharmacology of the novel benzimidazole-type FLAP inhibitor BRP-7 (**manuscript I**).
2) Establish a suitable cell-based model stably expressing 5-LO and FLAP in HEK293 cells to determine putative FLAP inhibitors and investigate the role of FLAP for LT biosynthesis *in cellulo* (**manuscript II**).
3) Study the influence of FLAP on 5-LO activity, metabolite profile, and subcellular localization of 5-LO (**manuscript II**).
4) Determine the time-resolved *in situ* protein-protein interaction of native 5-LO and FLAP in human primary leukocytes and HEK293 cells in relation to 5-LO activity and 5-LO translocation (**manuscript III**).
5) Identify modulating factors of the *in situ* assembly of 5-LO and FLAP at the nuclear membrane, and of its pharmacological inhibition (**manuscript III**).

3. MANUSCRIPTS

Manuscript I

Pergola, C., J. Gerstmeier, B. Monch, B. Caliskan, S. Luderer, C. Weinigel, D. Barz, J. Maczewsky, S. Pace, A. Rossi, L. Sautebin, E. Banoglu and O. Werz (2014). "The novel benzimidazole derivative BRP-7 inhibits leukotriene biosynthesis *in vitro* and *in vivo* by targeting 5-lipoxygenase-activating protein (FLAP)." **Br J Pharmacol** 171(12): 3051-3064.

Manuscript II

Gerstmeier, J., C. Weinigel, D. Barz, O. Werz and U. Garscha (2014). "An experimental cell-based model for studying the cell biology and molecular pharmacology of 5-lipoxygenase-activating protein in leukotriene biosynthesis." **Biochim. Biophys. Acta** 1840(9): 2961-2969.

Manuscript III

Gerstmeier, J., C. Weinigel, S. Rummler, O. Rådmark, O. Werz and U. Garscha (2015). "Time-resolved in situ assembly of the leukotriene-synthetic 5-LO / FLAP complex in blood leukocytes." **FASEB J**, under review after invitation to submit.

3.1 Manuscript I

The novel benzimidazole derivative BRP-7 inhibits leukotriene biosynthesis *in vitro* and *in vivo* by targeting 5-lipoxygenase-activating protein (FLAP)

Pergola, C., J. Gerstmeier, B. Monch, B. Caliskan, S. Luderer, C. Weinigel, D. Barz, J. Maczewsky, S. Pace, A. Rossi, L. Sautebin, E. Banoglu and O. Werz (2014)

British Journal of Pharmacology 171(12): 3051-3064.

Here, we describe the *in vitro* and *in vivo* pharmacology of the novel and highly potent direct FLAP inhibitor BRP-7 [1-(2-chlorobenzyl)-2-(1-(4-isobutylphenyl)ethyl)-1H-benzimidazole]. The benzimidazol derivative potently suppressed LT formation in primary intact human neutrophils (IC_{50} = 150 nM) and monocytes (IC_{50} = 40 nM), comcomitant with an impaired co-localization of 5-LO and FLAP. A reduced potency of BRP-7 to inhibit 5-LO product formation was observed in cell-free assays and upon ample supply of exogenous substrate. BRP-7 selectively bound to FLAP in pull-down assays, and did not interfere with other enzymes of the AA cascade like COX-1, mPGS-1, and $cPLA_2$-α. Finally, BRP-7 potently inhibited LT biosynthesis in HWB, and in a rat pleurisy and a mouse peritonitis model *in vivo*. Together, BRP-7 significantly abolishes 5-LO activity by interference with FLAP and exhibits anti-inflammatory activity *in vitro* and *in vivo*, with promising potential for further development.

Contribution (50%): Cell culture and performance of blood cell isolation, purification of recombinant human 5-LO from E.coli, analysis of [^{3}H]-AA release, evaluation of BRP-7 derivatives as FLAP inhibitors, HPLC analysis of 5-LO products, mPGS-1 assay, immunofluorescence microscopy of 5-LO in monocytes, analysis of data and preparation of graphs, analysis of statistics, co-writing of the manuscript.

BJP British Journal of Pharmacology

DOI:10.1111/bph.12625
www.brjpharmacol.org

RESEARCH PAPER

The novel benzimidazole derivative BRP-7 inhibits leukotriene biosynthesis *in vitro* and *in vivo* by targeting 5-lipoxygenase-activating protein (FLAP)

C Pergola[1], J Gerstmeier[1], B Mönch[1], B Çalışkan[2], S Luderer[3], C Weinigel[4], D Barz[4], J Maczewsky[1,5], S Pace[1,5], A Rossi[5], L Sautebin[5], E Banoglu[2] and O Werz[1]

[1]*Institute of Pharmacy, Friedrich-Schiller-University Jena, Jena, Germany,* [2]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Gazi University, Ankara, Turkey,* [3]*Department of Pharmaceutical Analytics, Pharmaceutical Institute, Eberhard-Karls-University Tuebingen, Tuebingen, Germany,* [4]*Institute of Transfusion Medicine, University Hospital Jena, Jena, Germany, and* [5]*Department of Pharmacy, University of Naples Federico II, Naples, Italy*

Correspondence
Dr Carlo Pergola, Institute of Pharmacy, Friedrich-Schiller-University Jena, Philosophenweg 14, D-07743 Jena, Germany.
E-mail: pergolacarlo@gmail.com

Keywords
5-lipoxygenase; 5-lipoxygenase activating protein; leukotriene; benzimidazole; inflammation

Received
4 November 2013
Revised
25 January 2014
Accepted
3 February 2014

BACKGROUND AND PURPOSE

Leukotrienes (LTs) are inflammatory mediators produced via the 5-lipoxygenase (5-LOX) pathway and are linked to diverse disorders, including asthma, allergic rhinitis and cardiovascular diseases. We recently identified the benzimidazole derivative BRP-7 as chemotype for anti-LT agents by virtual screening targeting 5-LOX-activating protein (FLAP). Here, we aimed to reveal the *in vitro* and *in vivo* pharmacology of BRP-7 as an inhibitor of LT biosynthesis.

EXPERIMENTAL APPROACH

We analysed LT formation and performed mechanistic studies in human neutrophils and monocytes, in human whole blood (HWB) and in cell-free assays. The effectiveness of BRP-7 *in vivo* was evaluated in rat carrageenan-induced pleurisy and mouse zymosan-induced peritonitis.

KEY RESULTS

BRP-7 potently suppressed LT formation in neutrophils and monocytes and this was accompanied by impaired 5-LOX co-localization with FLAP. Neither the cellular viability nor the activity of 5-LOX in cell-free assays was affected by BRP-7, indicating that a functional FLAP is needed for BRP-7 to inhibit LTs, and FLAP bound to BRP-7 linked to a solid matrix. Compared with the FLAP inhibitor MK-886, BRP-7 did not significantly inhibit COX-1 or microsomal prostaglandin E_2 synthase-1, implying the selectivity of BRP-7 for FLAP. Finally, BRP-7 was effective in HWB and impaired inflammation *in vivo*, in rat pleurisy and mouse peritonitis, along with reducing LT levels.

CONCLUSIONS AND IMPLICATIONS

BRP-7 potently suppresses LT biosynthesis by interacting with FLAP and exhibits anti-inflammatory effectiveness *in vivo*, with promising potential for further development.

Abbreviations

12-HHT, 12-hydroxy-5,8,10-heptadecatrienoic acid; 5-HPETE, 5-hydroperoxyeicosatetraenoic acid; 5-LOX, 5-lipoxygenase; AA, arachidonic acid; $cPLA_2$, cytosolic PLA_2; cysLTs, cysteinyl LTs; DPPH, 1,1-diphenyl-2-picrylhydrazyl; FLAP, 5-lipoxygenase-activating protein; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; hERG, human ether-a-go-go gene; HWB, human whole blood; IFM, immunofluorescence microscopy; mPGES-1, microsomal PGE_2 synthase-1; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; PGC buffer, PBS pH 7.4 containing 1 mg·mL^{-1} glucose and 1 mM $CaCl_2$; SDS-b, SDS-PAGE sample loading buffer; STI, soybean trypsin inhibitor

Introduction

The leukotrienes (LTs) comprise two different classes of pro-inflammatory lipid mediators derived from arachidonic acid (AA) with distinct biological activities. Whereas LTB_4 acts as a chemoattractant for leukocytes and promotes immunological responses, the cysteinyl-containing LT_s (cysLTs) C_4, D_4 and E_4 induce bronchoconstriction and mucus secretion, cause plasma extravasation and stimulate fibrocyte proliferation (Peters-Golden and Henderson, 2007). Consequently, intervention with LTs represents a pertinent pharmacological approach against inflammatory diseases, and anti-LT therapy has been validated in clinical trials of asthma and allergic rhinitis, with potential in other respiratory and allergic disorders (Peters-Golden and Henderson, 2007), as well as in cardiovascular diseases such as atherosclerosis, myocardial infarction, stroke and abdominal aortic aneurysm (Riccioni and Back, 2012).

The biosynthesis of LTs requires first the liberation of AA from membrane phospholipids that is conferred by the cytosolic PLA_2 ($cPLA_2$) (Uozumi *et al.*, 1997). Then, 5-lipoxygenase (5-LOX) catalyses the incorporation of molecular oxygen into AA to generate 5-hydroperoxyeicosatetraenoic acid (5-HPETE) that is dehydrated by 5-LOX into LTA_4 (for nomenclature see Alexander *et al.*, 2013). This unstable epoxide intermediate is further metabolized to bioactive LTs by LTA_4 hydrolase (yielding LTB_4) or LTC_4 synthase (yielding LTC_4) (Radmark *et al.*, 2007). Substantial experimental evidence indicates that the cellular production of LTs from endogenous AA requires the so-called 5-LOX-activating protein (FLAP), which may play an essential role in AA transfer to 5-LOX (Abramovitz *et al.*, 1993; Ferguson *et al.*, 2007). FLAP is a trimeric 18 kD protein localized at the nuclear membrane and belongs to the superfamily of membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG). However, neither an enzymatic function nor modulation by GSH has been revealed for FLAP thus far (Evans *et al.*, 2008; Ferguson, 2012). Apparently, FLAP acts as a scaffold for 5-LOX at the nuclear envelope (Bair *et al.*, 2012), where it facilitates access of AA to 5-LOX (Ferguson *et al.*, 2007). Importantly, genetic ablation or pharmacological interference of FLAP fully abolishes the generation of 5-LOX-derived products (Byrum *et al.*, 1997; Evans *et al.*, 2008), implying its crucial role in LT biosynthesis.

In the past, two chemotypes of FLAP inhibitors, namely the indole series (e.g. MK886) and quinoline-based compounds (e.g. BAY X-1005/DG-031) or hybrids thereof (MK591), have been developed and evaluated in clinical trials, but these compounds were not further explored (Evans *et al.*, 2008; Sampson, 2009; Ferguson, 2012). Recent re-assessment of FLAP inhibitors of the indole series provided GSK2190915 as a promising candidate that is currently undergoing phase II trials in patients with asthma (Stock *et al.*, 2011; Bain *et al.*, 2013; Follows *et al.*, 2013; Kent *et al.*, 2013; Snowise *et al.*, 2013). Also, the anti-inflammatory compound licofelone, which was originally developed as a dual inhibitor of the COX and 5-LOX pathways (Laufer *et al.*, 1994a,b), targets FLAP (Fischer *et al.*, 2007) and has reached clinical phase III for osteoarthritis (Raynauld *et al.*, 2009).

Inspired by the therapeutic potential of FLAP inhibitors, we attempted to discover novel chemotypes using a virtual screening approach targeting FLAP. By means of a combined ligand- and structure-based pharmacophore model, we recently identified BRP-7 [1-(2-chlorobenzyl)-2-(1-(4-isobutylphenyl)ethyl)-1*H*-benzimidazole] (Figure 1A) as a LT synthesis inhibitor in intact neutrophils, without direct effects on 5-LOX (Banoglu *et al.*, 2012). Here, we show that (i) BRP-7 inhibits 5-LOX product synthesis with typical features of well-recognized FLAP inhibitors; (ii) is effective in human whole blood (HWB); and (iii) has anti-inflammatory effects in two LT-related functional *in vivo* models, suggesting promising potential for further development.

Methods

Materials

BRP-7 was synthesized and characterized as reported previously (Banoglu *et al.*, 2012). RSC-3388 (*N*-{(2*S*,4*R*)-4-(biphenyl-2-ylmethyl-isobutyl-amino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl}-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl]acrylamide) was purchased from Calbiochem (Bad Soden, Germany), zileuton from Sequoia Research Products (Oxford, UK), MK886 from Cayman Chemical (Ann Arbor, MI, USA), and zymosan and λ-carrageenan type IV isolated from *Gigartina aciculairis* and *Gigartina pistillata* from Sigma (Milan, Italy). HPLC solvents were from VWR International GmbH (Darmstadt, Germany). AA, Ca^{2+} ionophore A23187, celecoxib, LPS, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), indomethacin and all other fine chemicals were from Sigma (Taufkirchen, Germany), unless stated otherwise. BRP-7 probes for fishing approach were synthesized as indicated in the Supporting Information Appendix S1.

Cells

Neutrophils and monocytes were isolated from buffy coats from adult healthy volunteers obtained at the Institute of Transfusion Medicine, University Hospital Jena. Neutrophils were immediately isolated by dextran sedimentation and centrifugation on Nycoprep cushions (PAA Laboratories, Linz, Austria) and hypotonic lysis of erythrocytes as described previously (Pergola *et al.*, 2008). Monocytes were separated from peripheral blood mononuclear cells by adherence to culture flasks as described previously (Pergola *et al.*, 2011). To exclude any cytotox effects of the test compounds, cell viability was analysed by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay as described previously (Tretiakova *et al.*, 2008).

Determination of 5-LOX product formation

For assays in intact cells, neutrophils or monocytes [5×10^6 and 2×10^6 mL^{-1}, respectively; in PBS (pH 7.4) containing 1 $mg \cdot mL^{-1}$ glucose and 1 mM $CaCl_2$ (PGC buffer); incubation volume, 1 mL] were pre-incubated with the compounds or vehicle (0.1% DMSO) for 15 min at 37°C. Then, 2.5 μM A23187 plus AA was added. For neutrophils, 40 μM AA was used as a standard concentration, as in previous studies (Werz *et al.*, 2002), whereas 10 μM AA was used for monocytes, because of substrate inhibition at higher AA concentrations. The reaction was stopped after 10 min with 1 mL methanol

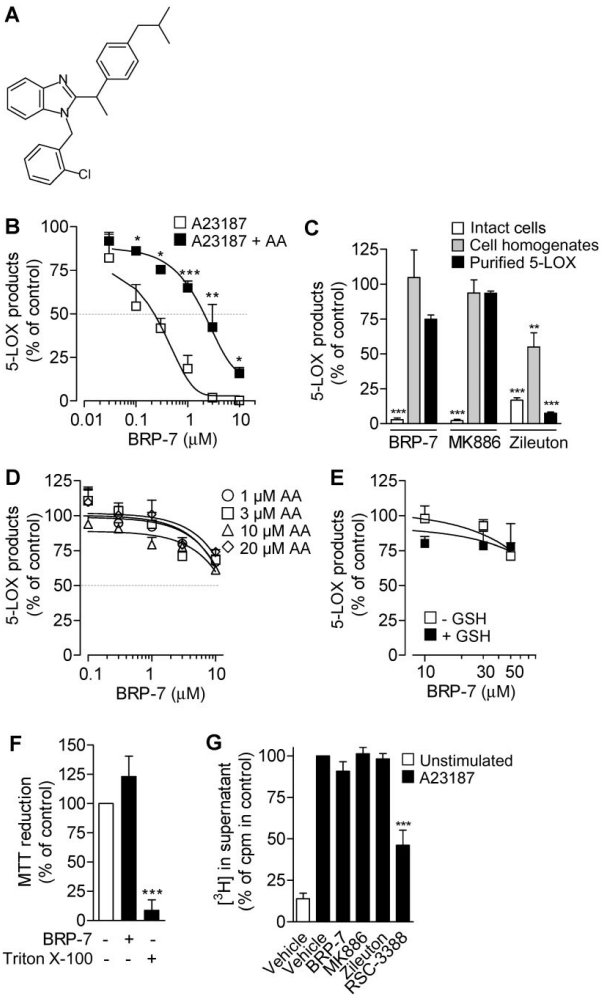


Figure 1

BRP-7 has features resembling other FLAP inhibitors. (A) BRP-7 structure. (B) 5-LOX product formation in human neutrophils stimulated with A23187 (2.5 μM), without or with AA (40 μM), after pre-incubation with BRP-7 or vehicle (0.1% DMSO). (C) Effects of 10 μM BRP-7, 100 nM MK886 or 10 μM zileuton on 5-LOX product formation in intact neutrophils and in neutrophil homogenates, and on the activity of human recombinant 5-LOX. Intact neutrophils were stimulated with A23187 (2.5 μM). For homogenates and purified 5-LOX, test compounds were added 5 min before the addition of 1 mM Ca^{2+} plus 40 μM AA. (D) Effects of BRP-7 on the activity of human recombinant 5-LOX at increasing AA substrate concentrations. (E) Reducing conditions do not restore 5-LOX inhibition by BRP-7. GSH (5 mM) or vehicle was added to homogenates of neutrophils, prior to BRP-7 addition. After 10 min on ice, 1 mM $CaCl_2$ and 40 μM AA were added for 10 min at 37°C. The 100% controls correspond to 5-LOX products in (B, C, E) intact cells, 117.5 ± 17.5 and 831.3 ± 50.9 ng·mL^{-1}, for A23187 or A23187 + AA, respectively; purified 5-LOX, 947.8 ± 125.2 ng·mL^{-1}; homogenates, 484.7 ± 72 ng·mL^{-1} and 429.6 ± 111.8 ng·mL^{-1}, without GSH and after addition of 5 mM GSH, respectively; (D) 37.9 ± 6.1, 153.1 ± 4.6, 610.3 ± 37.8 and 812.3 ± 22.6 ng·mL^{-1}, in the presence of 1, 3, 10 and 20 μM AA respectively. (F) Cell viability of human neutrophils after 30 min of pre-incubation at 37°C with vehicle (0.1% DMSO), 10 μM BRP-7 or 1% Triton X-100. (G) Analysis of radioactivity in supernatants of [^{3}H]-AA-labelled neutrophils after pre-incubation with 10 μM BRP-7, 100 nM MK886, 10 μM zileuton or 1 μM $cPLA_2$ inhibitor (RSC-3388) and stimulation with 2.5 μM A23187 plus 50 μM thiomersal; 100% corresponds to 18 073 ± 1735 cpm. Data are means + SEM; n = 3–9; *P < 0.05; **P < 0.01; ***P < 0.001 (B) versus inhibition by the corresponding BRP-7 concentration without the addition of AA; (C–F) versus 100% control; ANOVA plus Bonferroni.

and 30 µL 1 N HCl, and 200 ng PGB_1 and 500 µL of PBS were added.

For assays in cell homogenates, 1 mM EDTA was added to cells re-suspended in PBS. Samples were cooled on ice (5 min), sonicated (3 × 10 s) at 4°C, and 1 mM ATP and 1 mM DTT were added, as indicated. For assays with isolated 5-LOX, *Escherichia coli* BL21 was transformed with pT3-5LO plasmid, human recombinant 5-LOX protein was expressed at 30°C as described previously (Pergola *et al.*, 2012), and partially purified 5-LOX was added to 1 mL of a 5-LOX reaction mix (PBS, pH 7.4, 1 mM EDTA, 1 mM ATP). Samples (0.5–2 µg of partially purified 5-LOX, resulting in about 1000 ng·mL^{-1} 5-LOX products in an activity test performed with 20 µM AA; or cell homogenates, corresponding to 5×10^6 and 2×10^6 cells·mL^{-1} per sample, for neutrophils and monocytes, respectively) were incubated for 10 min at 4°C with vehicle or test compounds, pre-warmed for 30 s at 37°C, and 2 mM $CaCl_2$ and the indicated concentrations of AA were added. The reaction was stopped as indicated for intact cells.

For assays in HWB, freshly withdrawn blood from healthy adult donors was obtained by venipuncture and collected in monovettes containing 16 IE heparin·mL^{-1}. Aliquots of 2 mL were primed with 1 µg·mL^{-1} LPS for 15 min at 37°C, followed by incubation with vehicle (0.1% DMSO) or test compounds for 15 min at 37°C, and stimulation with 1 µM fMLP for 15 min at 37°C. Samples were prepared for extraction of metabolites and then extracted and analysed by HPLC as described previously (Pergola *et al.*, 2012).

5-LOX products include LTB_4, its *all-trans* isomers and 5-HPETE. The cysteinyl leukotrienes (cysLTs) C_4, D_4 and E_4 were not detected. For the determination of cysLTs in supernatants of monocytes, a cysLT ELISA kit from Enzo Life Sciences International Inc. (Lörrach, Germany) was used.

Determination of ^{3}H-labelled AA release

Release of ^{3}H-labelled AA from neutrophils and monocytes was analysed as previously described (Fischer *et al.*, 2005). Briefly, freshly isolated cells were re-suspended at 1×10^7 in 1 mL of RPMI 1640 medium containing 5 nM [^{3}H]-AA (corresponding to 0.5 µCi·mL^{-1}, specific activity 200 Ci·mmol^{-1}) and incubated for 120 min at 37°C in 5% CO_2 atmosphere. Cells were then washed twice with PBS containing 1 mg·mL^{-1} glucose and 2 mg·mL^{-1} fatty acid-free bovine albumin to remove unincorporated [^{3}H]-AA. Labelled cells (2×10^7 mL^{-1} PMNL and 5×10^6 mL^{-1} monocytes) were re-suspended in 1 mL of PGC containing 2 mg·mL^{-1} fatty acid-free bovine albumin and pre-incubated with 0.1% DMSO or test compounds (15 min, 37°C). Neutrophils were stimulated with 2.5 µM A23187 in the presence of 50 µM thiomersal to block the re-acylation of unconverted AA into membranes (Zarini *et al.*, 2006), and monocytes were stimulated with 2.5 µM A23187. The samples were then placed on ice, centrifuged and aliquots (300 µL) of the supernatants were assayed for radioactivity by scintillation counting (Micro Beta Trilux; PerkinElmer, Waltham, MA, USA).

Analysis of subcellular redistribution of 5-LOX

For analysis of 5-LOX subcellular redistribution, neutrophils (3×10^7 mL^{-1} PGC buffer) were pre-incubated with 0.1% DMSO or BRP-7 for 15 min at 37°C and stimulated with 2.5 µM A23187. After 5 min, the reaction was stopped on ice and samples were centrifuged (200× *g* for 5 min at 4°C). Subcellular fractionation was performed by mild detergent (0.1% NP40) lysis, yielding a nuclear and a non-nuclear fraction as described previously (Werz *et al.*, 2001). Aliquots (20 µL) of nuclear and non-nuclear fractions were analysed by SDS-PAGE and Western blotting as described previously (Pergola *et al.*, 2012). 5-LOX antiserum (1551, AK7, kindly provided by Dr Olof Rådmark, Karolinska Institutet, Stockholm, Sweden) was used at 1:100 dilution; alkaline phosphatase-conjugated IgGs (Sigma-Aldrich) were used at 1:1000 dilution. Proteins were visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate in detection buffer [100 mM Tris/HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$].

Analysis of 5-LOX subcellular localization by indirect immunofluorescence microscopy (IFM) was performed as described previously (Pergola *et al.*, 2008; 2011). In brief, neutrophils or monocytes were pre-incubated with 0.1% DMSO or test compounds (15 min at 37°C), then placed onto poly-L-lysine (MW 150 000–300 000; Sigma-Aldrich)-coated glass coverslips, and activated by the addition of 2.5 µM A23187 for 3 min at 37°C. Cells were fixed in methanol (–20°C, 30 min) and permeabilized with 0.1% Tween 20 in PBS (RT, 10 min), followed by three washing steps with PBS. Samples were blocked with 10% non-immune goat serum (Invitrogen, Darmstadt, Germany) for 10 min at RT, washed with PBS and incubated with mouse anti-5-LOX (kindly provided by Dr Dieter Steinhilber, University of Frankfurt, Germany) and rabbit anti-FLAP (1:200; Abcam, Cambridge, UK) for 1 h at RT. The coverslips were washed 10 times with PBS, incubated for 10 min at RT in the dark with Alexa Fluor 488 goat anti-rabbit (1:1500) and Alexa Fluor 555 anti-mouse IgG (1:1000) (Invitrogen) diluted in PBS, and washed 10 times with PBS. Where indicated, the DNA was stained with 0.1 µg·mL^{-1} DAPI in PBS for 3 min at RT in the dark. The coverslips were then mounted on glass slides with Mowiol (Calbiochem) containing 2.5% *n*-propyl gallate (Sigma-Aldrich). The fluorescence was visualized with a Zeiss Axio Observer.Z1 microscope (Carl Zeiss, Jena, Germany) and a Plan-Apochromat 100×/1.40 Oil DIC M27 objective. Images were taken with an AxioCam MR3 camera and were acquired, cut, linearly adjusted in the overall brightness and contrast, and exported to TIF by the AxioVision 4.8 software (Carl Zeiss).

Immobilization of BRP-7 derivatives and pull-down assays

Toyopearl AF-Amino-650 M resin (Tosoh Bioscience, Stuttgart, Germany) was washed five times with water and acetate buffer (0.1 M sodium acetate, 0.5 M NaCl; pH 4) and once with 80% MeOH (pH 5). Then, 100 µmol of **1a** and **2a** carboxylate analogues were solubilized in 10 mL of 80% MeOH by adding 1 M NaOH until the compounds were completely dissolved. Subsequently, pH was adjusted to 5 with 1 M HCl. Toyopearl resin (500 µL) and 40 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide were added to form amides **1b** and **2b** (48 h, pH 5). Probes were washed with acetate buffer and stored in 20% MeOH until use.

For preparation of nuclear membranes, 1.5×10^9 neutrophils were lysed by a mild detergent (0.1% NP40) lysis with 1.6 mL of 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^{-1} soybean trypsin inhibitor (STI) and 10 μg·mL^{-1} leupeptin], vortexed (3 × 5 s), kept on ice for 10 min and centrifuged (1000× *g*, 10 min, 4°C) (Werz *et al.*, 2001). The resulting pellets (nuclear fractions) were re-suspended in 500 μL of TKM buffer [50 mM Tris–HCl (pH 7.4), 25 mM KCl, 5 mM $MgCl_2$, 250 mM sucrose, 1 mM EDTA, 1 mM PMSF, 60 μg·mL^{-1} STI and 10 μg·mL^{-1} leupeptin]. Nuclei were disrupted by sonication (3 × 5 s), samples were cleared by centrifugation (5 min, 10 000× *g*, 4°C) and the resulting supernatants were centrifuged for 1 h at 100 000× *g*, for isolation of membranes. The pellet was then re-suspended in 100 μL of membrane buffer [100 mM Tris–HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 5% glycerol, 0.05% Tween 20, 1 mM PMSF, 60 μg·mL^{-1} STI, 10 μg·mL^{-1} leupeptin], resulting in a protein concentration of about 6.5 mg·mL^{-1}.

For pull-down assays, 50 μL of resin was added to 500 μL of binding buffer [50 mM HEPES (pH 7.4), 200 mM NaCl, 1 mM EDTA] and 8 μL of the neutrophil nuclear membranes (corresponding to about 50 μg of protein), incubated (2.5 h, 4°C) and washed three times with 500 μL of binding buffer. Proteins were eluted with 50 μL of 2× SDS-b (SDS-PAGE sample loading buffer) (5 min, 96°C). Equal amounts of protein were loaded onto 16% acrylamide gels and analysed for FLAP by Western blotting using rabbit anti-FLAP (Abcam), anti-rabbit IRDye 800CW (Li-Cor Biosciences, Lincoln, NE, USA), and detection with an Odyssey Infrared Imaging System (Li-Cor Bioscience), and analysis by the Odyssey application software (version 3.0.25).

Activity assays of COX-1 and -2, of microsomal PGE_2 synthase-1 (mPGES-1)

Activities of the purified ovine COX-1 (50 units) or human recombinant COX-2 (20 units) (both from Cayman Chemical) were analysed as described previously (Koeberle *et al.*, 2008). Briefly, the COX enzymes were pre-incubated with the test compound for 5 min at 4°C, samples were pre-warmed for 60 s at 37°C, AA (5 μM for COX-1, 2 μM for COX-2) was added, and after 5 min at 37°C, the COX product 12-hydroxy-5,8,10-heptadecatrienoic acid (12-HHT) was analysed by HPLC. Indomethacin (10 μM) was used as a reference inhibitor.

To analyse the effect of BRP-7 on cellular COX-1 activity, freshly isolated human platelets (10^8 mL^{-1} PGC buffer) were pre-incubated with test compounds for 15 min at 37°C and stimulated for 10 min at 37°C with 5 μM AA. The COX reaction was stopped and 12-HHT was analysed as for the isolated enzyme.

To analyse the effect of BRP-7 on cellular COX-2 activity, A549 cells were stimulated with 2 ng·mL^{-1} IL-1β for 72 h to induce COX-2 expression, washed twice with PBS, re-suspended in PGC buffer (2 × 10^6 cells·mL^{-1}) and pre-incubated with the indicated compounds for 15 min at 37°C. After stimulation for 15 min at 37°C with 3 μM AA, the reaction was stopped on ice, the supernatants were recovered after centrifugation at 60× *g* for 10 min at 4°C, and 6-keto $PGF_{1\alpha}$ formation was measured by ELISA (6-keto $PGF_{1\alpha}$ from Sapphire Bioscience, Waterloo, Australia).

Preparation of A549 cells and determination of mPGES-1 activity was performed as described previously (Koeberle *et al.*, 2008). In brief, cells (2 × 10^6 cells in 20 mL of medium) were plated, incubated for 16 h (37°C, 5% CO_2) and then the culture medium was replaced by fresh DMEM/high glucose (4.5 g·L^{-1}) medium containing 2% (v v^{-1}) FCS. mPGES-1 expression was induced by 2 ng·mL^{-1} IL-1β for 72 h. Cells were sonicated and the microsomal fraction was prepared by differential centrifugation at 10 000× *g* for 10 min and at 174 000× *g* for 1 h. The pellet was re-suspended in 1 mL of homogenization buffer [0.1 M potassium phosphate buffer (pH 7.4), 1 mM PMSF, 60 μg·mL^{-1} STI, 1 μg·mL^{-1} leupeptin, 2.5 mM glutathione and 250 mM sucrose] and then serially diluted in 0.1 M potassium phosphate buffer (pH 7.4) containing 2.5 mM glutathione for activity test. A dilution leading to about 10 μM PGE_2 (using 20 μM PGH_2 as substrate; incubation volume, 100 μL) was finally used for the experiments. The microsomal membranes (total volume, 100 μL) were pre-incubated with the test compounds or 0.1% DMSO. After 15 min, PGE_2 formation was initiated by the addition of PGH_2 (20 μM). After 1 min at 4°C, the reaction was terminated with 100 μL of stop solution (40 mM $FeCl_2$, 80 mM citric acid and 10 μM of 11β-PGE_2 as internal standard), and PGE_2 was separated by solid-phase extraction (RP-18 material) and analysed by HPLC.

Evaluation of human ether-a-go-go gene (hERG) and CYP3A4 inhibition

The experiments for evaluation of hERG and cytochrome P450 (CYP3A4) inhibition were performed by Cyprotex Discovery Ltd (Macclesfield, UK), as indicated in the Supporting Information Appendix S1. Briefly, the hERG assay was performed with CHO cells stably transfected with hERG and electrophysiology measurements by an IonWorks™ HT instrument (Molecular Devices Corporation, Sunnyvale, CA, USA) and specialized multi-well plate (PatchPlate™). For CYP3A4 assay, the test compound was incubated with human liver microsomes and NADPH in the presence of the specific CYP3A4 substrate, midazolam. 1-Hydroxymidazolam was monitored by LC-MS/MS.

Animal studies

The animal studies are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). Male Wistar Han rats (200–230 g; Harlan, San Pietro al Natisone, Italy, and Charles River, Calco, Italy) and male CD-1 mice (35–40 g; Charles River) (30 rats, 50 mice) were housed at the Department of Pharmacy (Naples, Italy) in a controlled environment (21 ± 2°C) and provided with standard rodent chow and water. Animals were allowed to acclimatize for 4 days before the experiments and were subjected to a 12 h light–12 h dark schedule. Experiments were conducted during the light phase. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (Ministerial Decree 116/92) as well as with the European Economic Community regulations (Official Journal of the European Community L 358/1 12/18/1986). The animal studies were approved by the local ethical committee of the University of Naples Federico II on 10 February 2011 (approval number 2011/0017635) and on 28 June 2011 (approval number 2011/0075376).

The carrageenan-induced pleurisy in rats was performed as described previously (Pergola *et al.*, 2012). Thus, BRP-7 or MK886 at the indicated dose or vehicle (1.5 mL of 0.9% saline solution containing 4% DMSO) was given i.p. 30 min before λ-carrageenan type IV 1% (w v^{-1}; 0.2 mL), which was injected into the pleural cavity. The animals were killed by inhalation of CO_2 at 4 h, the pleural exudates were collected, the cells in the exudates were counted and the amounts of LTB_4 were assayed by EIA kit (Cayman Chemical), according to manufacturer's instructions.

For zymosan-induced peritonitis in mice, BRP-7 or MK-886 at the indicated dose or vehicle (0.5 mL of 0.9% saline solution containing 2% DMSO) was given i.p. 30 min before zymosan i.p. injection (0.5 mL of suspension of 2 mg mL^{-1} in 0.9% w/v saline). Mice were killed by inhalation of CO_2 at the indicated time, followed by a peritoneal lavage with 3 mL of cold PBS. Exudates were collected, the cells in the exudates were counted and LTC_4 was measured by EIA (Cayman Chemical). Vascular permeability was assessed by the Evans blue method according to Kolaczkowska *et al.* (2002).

Statistics

Results are expressed as mean + SEM of *n* observations, where *n* represents the number of experiments performed on different days in duplicates or the number of animals, as indicated. The IC_{50} values were determined by interpolation on semi-logarithmic graphs and validated with GraphPad Prism software (GraphPad, San Diego, CA, ISA). Statistical evaluation of the data was performed by one-way ANOVA) for independent or correlated samples, followed by Tukey's HSD (honestly significant difference) *post hoc* tests. Where appropriate, Student's *t*-test was applied. A *P*-value < 0.05 (*) was considered significant.

Results

Differential inhibition of 5-LOX product synthesis by BRP-7 in cell-based and cell-free assays

Firstly, we studied the ability of BRP-7 to interfere with 5-LOX product synthesis and we addressed whether BRP-7 shares typical mechanistic properties with FLAP inhibitors. As we observed no significant differences in the inhibitory potency between the individual enantiomers (Sardella *et al.*, 2013), racemic BRP-7 was used. In agreement with our previous results (Banoglu *et al.*, 2012), BRP-7 concentration-dependently inhibited the formation of 5-LOX-derived products (i.e. LTB_4 and its *trans* isomers and 5-HPETE) in A23187-stimulated human neutrophils (IC_{50} = 0.15 ± 0.08 μM; Figure 1B). However, BRP-7 did not inhibit 5-LOX product formation in cell-free assays (i.e. human recombinant 5-LOX or neutrophil homogenates), which was similar to the FLAP inhibitor MK886, whereas the direct 5-LOX inhibitor zileuton was active in this respect (Figure 1C). Failure to inhibit 5-LOX in such cell-free assays was independent of the substrate concentration (Figure 1D), excluding a competitive mode of action, and may indicate the lack of a direct effect on 5-LOX, but it may also be related to a lack of effect under non-reducing conditions, as reported for non-redox-type 5-LOX inhibitors (Werz *et al.*, 1998). In contrast to this class of compounds, however, inclusion of GSH in homogenates did not restore the inhibitory effect of BRP-7 on 5-LOX (Figure 1E). Also, BRP-7 was ineffective as a radical scavenger in a DPPH (1,1-diphenyl-2-picrylhydrazyl) assay (not shown), excluding interference by redox-related mechanisms. Together, direct interference of BRP-7 with 5-LOX can be excluded. Moreover, non-specific effects of BRP-7 in intact cells due to cell toxicity are unlikely because BRP-7 had no significant effects on cell viability (Figure 1F). Of interest, as described for MK886 (Fischer *et al.*, 2007), the efficiency of BRP-7 in A23187-stimulated neutrophils was significantly reduced when excess (40 μM) exogenous AA was included (Figure 1A), despite it not inducing a significant inhibition of AA release from [^{3}H]-AA-labelled neutrophils (Figure 1G).

BRP-7 blocks 5-LOX co-localization with FLAP at the nuclear envelope in neutrophils

FLAP inhibitors were shown to block 5-LOX translocation to the nucleus seemingly by preventing the association between 5-LOX/FLAP (Evans *et al.*, 2008). As shown in Figure 2A, upon stimulation of neutrophils with A23187, cytosolic 5-LOX is translocated to the nucleus, and this was concentration-dependently inhibited by BRP-7, starting at 1 μM, although complete inhibition was not observed even at 10 μM. IFM allowed more detailed analysis of 5-LOX subcellular localization and also of FLAP (Figure 2B). In unstimulated cells, FLAP was localized to the nuclear envelope, whereas 5-LOX was distributed within the cytosol, and co-localization of 5-LOX and FLAP was not evident. Upon A23187 stimulation, 5-LOX clearly co-localized with FLAP at the nuclear envelope. Pre-incubation of neutrophils with BRP-7 or MK886 (10 μM and 100 nM, concentrations that completely inhibited 5-LOX product formation) prevented the 5-LOX association with FLAP, although only to a partial extent, indicating that inhibition of 5-LOX translocation by FLAP inhibitors is not directly related to LT suppression.

To analyse the interaction of BRP-7 with FLAP, we used an affinity chromatography method using an insoluble BRP-7 matrix and solubilized FLAP (from neutrophil membranes) as the protein source. Thus, the 2'-chlorine in BRP-7 was replaced by a methylene ether linked either via an amide function to a sepharose matrix (yielding matrix **1b**) or to ethyl carboxylate (yielding **1a**) (Figure 3A). Compound **1a** inhibited 5-LOX product synthesis, although with a lower potency as compared to BRP-7 (IC_{50} for **1a** = 2.6 ± 1.1 μM). The truncated analogues thereof, that is, matrix **2b** and compound **2a**, were used as negative, inactive controls (Figure 3A; IC_{50} for **2a** was >10 μM). Interestingly, FLAP was enriched in pull-downs using matrix **1b** that carries the bioactive probe, as compared to matrix **2b** (Figure 3B). Excess BRP-7 (100 μM) prevented the FLAP enrichment in pull-downs with matrix **1b** (Figure 3B), as expected. 5-LOX protein was not detected in the pull-downs (not shown), excluding possible indirect effects on FLAP.

Cellular pharmacology of BRP-7 in monocytes

Besides neutrophils, monocytes are a major source of LTs (Surette *et al.*, 1993; Pergola *et al.*, 2011). We thus determined

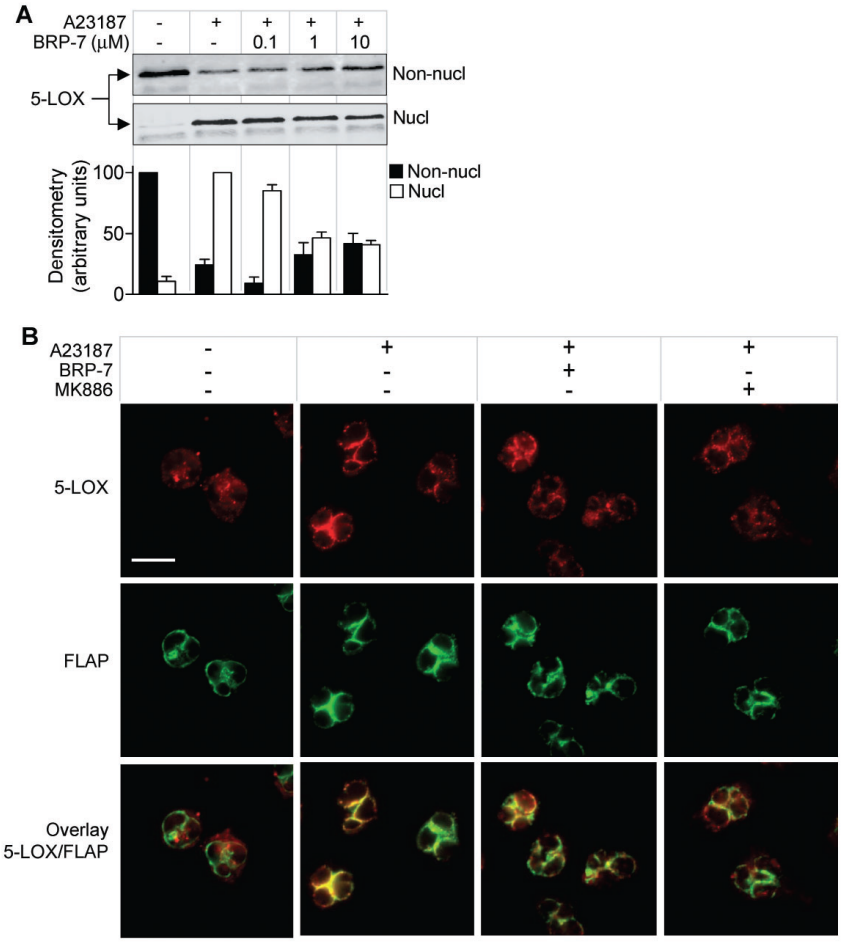


Figure 2

BRP-7 reduces the nuclear translocation of 5-LOX in human neutrophils. Analysis of 5-LOX localization by (A) subcellular fractionation and (B) IFM. (A) Neutrophils were pre-incubated for 15 min at 37°C with vehicle (0.1% DMSO) or BRP-7 and then stimulated with 2.5 μM A23187 for 5 min at 37°C and lysed by mild detergent (0.1% NP-40). Non-nuclear (non-nucl) and nuclear (nucl) fractions were analysed by Western blot and densitometric analysis was performed. Data are given as means + SEM; $n = 3$. (B) Neutrophils were pre-incubated with vehicle (0.1% DMSO), BRP-7 (10 μM), MK-886 (100 nM) and stimulated by A23187 (2.5 μM; 3 min). Cells were fixed, permeabilized and incubated with mouse anti-5-LOX and rabbit anti-FLAP, followed by Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 555 goat anti-mouse IgG. Red: 5-LOX; green: FLAP. Scale bar: 10 μm. The pictures shown are representative of three similar samples.

the effects of BRP-7 on 5-LOX product formation in human monocytes stimulated with A23187 in the presence or absence of exogenous AA (10 μM). BRP-7 potently inhibited the formation of 5-LOX products (LTB_4 and its *trans* isomers and 5-HPETE) in the absence of AA with an IC_{50} = 0.04 ± 0.01 μM (Figure 4A). In the presence of 10 μM AA, BRP-7 still inhibited 5-LOX activity, but, similar to that observed for neutrophils, with lower potency (IC_{50} = 0.25 ± 0.1 μM). In contrast to neutrophils, monocytes may convert LTA_4 by LTC_4 synthase to LTC_4, and BRP-7 efficiently also suppressed the synthesis of cysLTs (IC_{50} = 0.03 ± 0.01 μM; Figure 4B). In monocyte homogenates, BRP-7 up to 0.3 μM failed to significantly inhibit 5-LOX activity and this was also found for MK886 but not for zileuton, as expected (Figure 4A). Analysis of cell viability revealed no compromising effect of BRP-7 (10 μM) as compared to staurosporine (1 μM), used as control

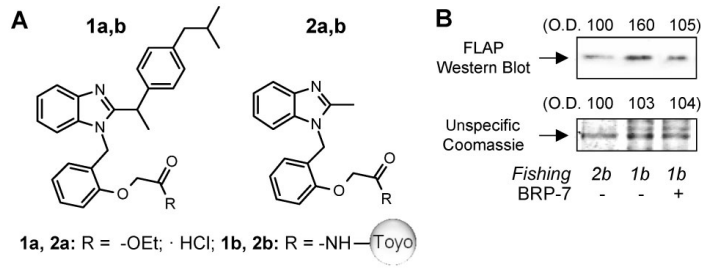


Figure 3

FLAP binds to an immobilized BRP-7 derivative. (A) Structures of BRP-7 derivatives used for the evaluation of IC_{50} values for 5-LOX products in A23187-stimulated human neutrophils (compounds **1a** and **2a**), and for protein fishing experiments after coupling on amino-activated Toyopearl AF-Amino-650 (compounds **1b** and **2b**). Compounds **2a** and **2b** were used as inactive controls. In (B), nuclear membrane proteins of human neutrophils were incubated with **1b** or **2b** functionalized beads. For **1b** functionalized beads, competition was performed with 100 μM BRP-7, as indicated. Precipitated proteins were analysed by Western blot for FLAP or by Coomassie staining followed by densitometry. Similar results were obtained in two additional experiments.

(Figure 4C). In addition to 5-LOX products, monocytes also generate other related eicosanoids from AA including 12- and 15-HPETE, as well as the COX product 12-HHT (Pergola *et al.*, 2011). However, neither the formation of 12- and 15-HPETE, nor 12-HHT, nor the amount of radioactivity in the supernatant (from [^{3}H]-AA) after A23187 stimulation was affected by 10 μM BRP-7 (Figure 4D,E), implying it has a selective effect on 5-LOX product synthesis.

As observed previously (Pergola *et al.*, 2011), the bulk of the 5-LOX protein in resting monocytes was localized inside the nucleus, whereas FLAP was found at the nuclear membrane (Figure 4F). A23187 stimulation redistributed 5-LOX to the nuclear membrane, where it co-localized with FLAP. BRP-7 and MK886 efficiently prevented the co-localization of 5-LOX and FLAP, even though the compounds failed to restore the pattern to that of resting cells with clear intranuclear 5-LOX. These data indicate that FLAP inhibitors prevent the association of 5-LOX with FLAP in stimulated monocytes but do not generally interfere with the redistribution of 5-LOX in these stimulated cells.

Effects of BRP-7 on COX-1 and -2, mPGES-1, CYP3A4 and hERG

FLAP inhibitors such as MK886 tend also to inhibit other enzymes involved in the AA cascade, including COX-1 and mPGES-1 (Claveau *et al.*, 2003; Koeberle *et al.*, 2009), which let us to evaluate their selectivity. BRP-7 (at 10 μM) did not inhibit COX-1 or COX-2 in both cell-free (Figure 5A,B) and cellular (Figure 5C,D) assays, and also failed to significantly block mPGES-1 activity in a cell-free assay (Figure 5E). Reference compounds for COX enzymes (indomethacin) and for mPGES-1 (MK886) confirmed the functionality of the assays respectively. Finally, we tested the ability of BRP-7 to inhibit CYP3A4, the most common CYP-isoforms in drug metabolism for prediction of probable drug interactions. BRP-7 did not significantly inhibit CYP3A4 activity up to 25 μM as compared to the reference drug ketoconazole (which showed an IC_{50} of 0.04 μM; n = 7). In addition, BRP-7 as a lipophilic benzimidazole derivative could bind to the human ether-a-go-go gene (hERG) voltage-gated potassium channel thereby prolonging the drug-induced QT interval (Yao *et al.*, 2008). However, there was no appreciable hERG inhibition up to 10 μM (0.97% inhibition; n = 19).

Efficiency of BRP-7 in HWB and efficacy in vivo

Given the favourable efficiency and selectivity of BRP-7, we next aimed to estimate its effectiveness and pharmacological relevance under more complex systems and *in vivo*. Thus, we analysed 5-LOX product synthesis in HWB stimulated with the pathophysiological relevant stimuli LPS and fMLP, which includes important variables such as plasma protein binding, presence of co-factors and cell–cell interactions. BRP-7 significantly inhibited 5-LOX product synthesis in blood with an IC_{50} of 4.8 ± 1 μM (Figure 6A), which is slightly higher, in the single-digit micromolar range, than that for MK886 (IC_{50} of 1.1 ± 0.4 μM; not shown).

We next studied the effects of BRP-7 *in vivo* using the carrageenan-induced pleurisy in rats, an animal model of acute inflammation involving 5-LOX. The i.p. pretreatment (30 min before carrageenan administration) of rats with 10, 20 or 30 mg·kg^{-1} of BRP-7 significantly reduced the inflammatory reaction, measured as exudate volume, inflammatory cell numbers and LTB_4 levels in the pleural exudates (Table 1, Figure 6B), with an ID_{50} value for LTB_4 inhibition of about 20 mg·kg^{-1}. For comparison, the reference FLAP inhibitor MK886 (at the maximally effective dose of 1 mg·kg^{-1}, i.p.) also significantly reduced exudate volume, infiltrated cells and LTB_4 (Table 1, Figure 6B). In zymosan-induced peritonitis in mice, another well-recognized model of acute inflammation, BRP-7 significantly impaired levels of LTC_4 with an ID_{50} of about 20 mg·kg^{-1} (Figure 6C). At this dose, BRP-7 reduced vascular permeability by 38% and inhibited neutrophil infiltration by 30% (Table 2), compared to reductions of 76 and 37% by MK886 (1 mg·kg^{-1}), respectively, implying that BRP-7-induced inhibition of LT biosynthesis *in vivo* is associated with its anti-inflammatory efficacy.

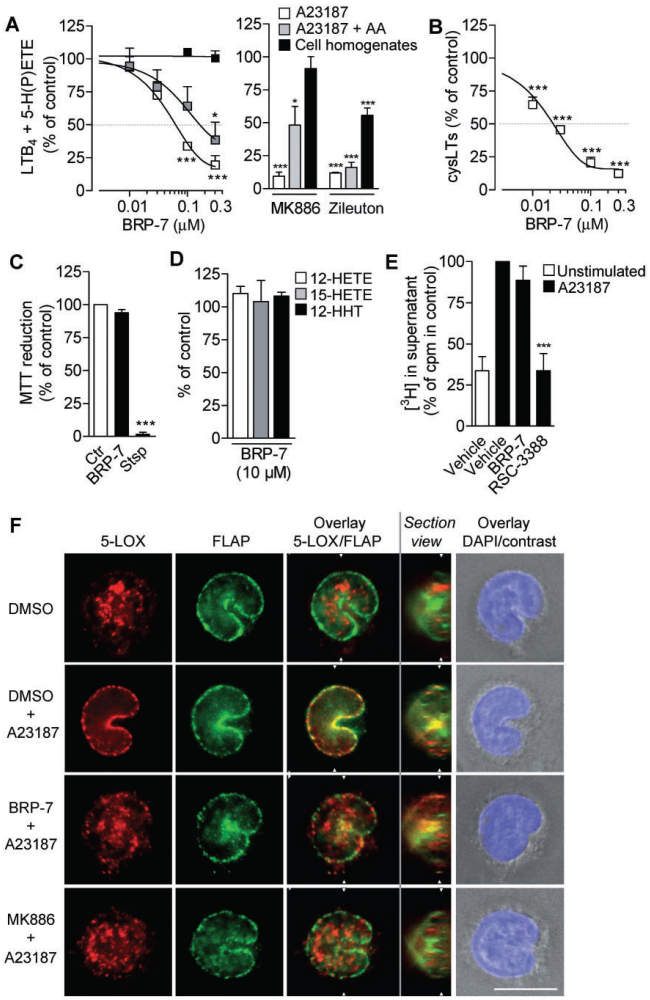


Figure 4

Cellular pharmacology of BRP-7 in human monocytes. (A, B) Effect of BRP-7, 100 nM MK886 or 10 μM zileuton on (A) LTB_4 + 5-H(P)ETE and (B) cysLT formation in intact human monocytes stimulated with A23187 (2.5 μM) in the presence or absence of exogenous AA (10 μM) or in monocyte homogenates treated with 1 mM Ca^{2+} plus 10 μM AA. Pre-incubation with compounds or vehicle (0.1% DMSO) was performed for 15 min at 37°C for intact cells or 5 min at 4°C for homogenates. The 100% controls correspond to (A) A23187, 42.0 ± 4.1 ng·mL^{-1}; A23187 + AA, 29.2 ± 1.3 ng·mL^{-1}; homogenates, 49.7 ± 13.2 ng·mL^{-1}; (B) 583 ± 85 pg·mL^{-1}. (C) Cell viability of human monocytes after a 30 min pre-incubation at 37°C with vehicle (0.1% DMSO), 10 μM BRP-7 or 1 μM staurosporine (stsp). (D) Effect of 10 μM BRP-7 on the formation of 12-HETE, 15-HETE and 12-HHT in monocytes stimulated for 10 min at 37°C with A23187 (2.5 μM). The 100% controls correspond to 12-HETE, 127.5 ± 27.2 ng·mL^{-1}; 15-HETE, 3.9 ± 1.2 ng·mL^{-1}; 12-HHT, 19.4 ± 2.6 ng·mL^{-1}. (E) Analysis of radioactivity in supernatants of [^{3}H]-AA-labelled monocytes after pre-incubation with 10 μM BRP-7 or 1 μM $cPLA_2$ inhibitor (RSC-3388) and stimulation with 2.5 μM A23187;100% corresponds to 25 437 ± 4226 cpm. (F) Analysis of 5-LOX localization by indirect IFM after pre-incubation of adherent monocytes for 15 min at 37°C with vehicle (0.1% DMSO), BRP-7 (10 μM) or MK-886 (100 nM) and stimulation with A23187 (2.5 μM, 3 min). Cells were fixed, permeabilized and incubated with mouse anti-5-LOX and rabbit anti-FLAP, followed by Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 555 goat anti-mouse IgG and DAPI. Red, 5-LOX; green, FLAP; blue, DNA. Scale bar: 10 μm. Sections were generated with the AxioVision 4.8 software from 0.2 μm step Z stacks of adherent monocytes (adhesion substrate on the right side of the view). The cutting plane is indicated in the overlays by white triangles. In (A)–(C), data are expressed as percentage of vehicle control (100%) and are means + SEM; $n = 3$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (A) versus 100% control; ANOVA plus Bonferroni.

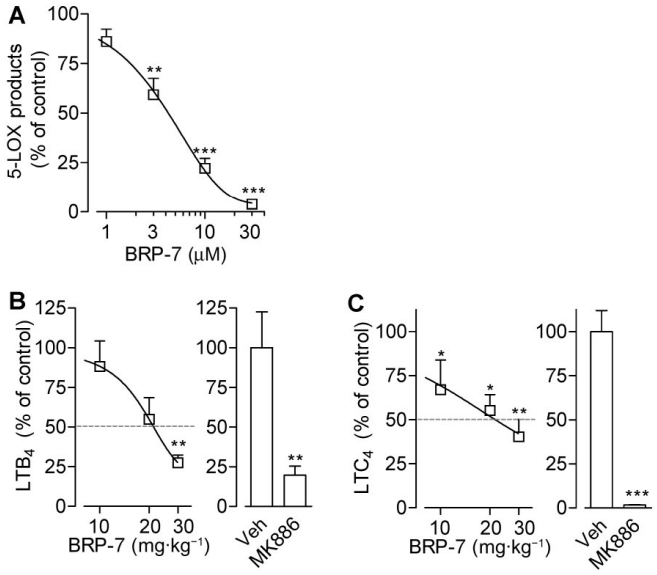


Figure 6

BRP-7 suppresses 5-LOX product formation in human whole blood. Effect of BRP-7 on (A) 5-LOX product formation in human whole blood stimulated with LPS (1 μg·mL^{-1}; 30 min, 37°C) and fMLP (1 μM; 15 min, 37°C). BRP-7 or vehicle (0.1% DMSO) were added 10 min before fMLP. 5-LOX products were assessed by HPLC and are means + SEM of percentage of vehicle control (100%, corresponding to 41.3 ± 6.5 ng·mL^{-1}, n = 6); (B) LTB_4 levels in carrageenan-induced pleurisy in rats; (C) LTC_4 levels in zymosan-induced peritonitis in mice. In (B) and (C), male rats or mice (n = 5–7, each group) were treated i.p. with 10, 20 or 30 mg·kg^{-1} BRP-7, 1 mg·kg^{-1} MK886 or vehicle (4 or 2% DMSO, for rat pleurisy and mouse peritonitis, respectively), 30 min before (B) intrapleural injection of carrageenan or (C) i.p. injection of zymosan. Analysis was performed (B) 4 h after carrageenan injection or (C) 30 min after zymosan injection. Data are means + SEM % of vehicle control; 100% corresponds to (B) 0.573 ± 0.130 ng·mL^{-1} LTB_4 or (C) 142.3 ± 17.1 ng·mL^{-1} LTC_4. **P < 0.01; ***P < 0.001 versus vehicle control; ANOVA plus Bonferroni.

Table 2

Effect of BRP-7 on zymosan-induced peritonitis in mice

Treatment[a]	LTC_4 (ng·mL^{-1})[b]	Vascular permeability (A_{650})[b]	Inflammatory cells (× 10^6)[c]
Vehicle	144.1 ± 20.0	1.35 ± 0.12	24.5 ± 1.9
BRP-7	70.4 ± 16.0*	0.84 ± 0.09*	17.7 ± 1.0**
	48.8%	*62.2%*	*69.7%*
MK886	7.5 ± 1.6***	0.32 ± 0.05***	16.1 ± 0.5**
	5.2%	*23.7%*	*63.4%*

Data are means ± SEM, n = 6. Values in italics are percentage of vehicle control.
[a]Male mice (n = 6, each group) were treated i.p. with 20 mg·kg^{-1} BRP-7, 1 mg·kg^{-1} MK886 or vehicle (2% DMSO), 30 min before i.p. injection of zymosan.
[b]Analysis was performed 30 min after zymosan injection.
[c]Analysis was performed 4 h after zymosan injection.
*P < 0.05; **P < 0.01; ***P < 0.001 versus vehicle (Student's t-test).

tion and this is accompanied by reduced LT levels. Therefore, BRP-7 might represent a novel basic structure for the development of alternative FLAP inhibitors suitable for exploitation as tools and/or potential therapeutics.

Pharmacological intervention with the LT pathway encompasses essentially three different strategies, that is, inhibition of enzymes involved in LT biosynthesis (e.g. 5-LOX, LTA_4 hydrolase or LTC_4 synthase), antagonism of LT

receptors (e.g. the cysLT$_1$ antagonist montelukast) and inhibition of LT formation by interference with FLAP. As compared to LT receptor antagonism and to inhibition of LTA$_4$ hydrolase or LTC$_4$ synthase, targeting 5-LOX or FLAP appears advantageous because it leads to suppression of both classes of LTs (LTB$_4$ and cysLT) and is appealing for its higher efficacy as an anti-inflammatory therapy. Although the possibility of direct inhibition of 5-LOX as a medical intervention has been extensively explored, the successful development of 5-LOX inhibitors has been strongly hampered due to their toxicity or lack of *in vivo* efficacy (Pergola and Werz, 2010), and until today, only one 5-LOX inhibitor (zileuton) could possibly enter the market.

In contrast, targeting FLAP has demonstrated promise in animal studies and in early clinical phases, but no FLAP inhibitor has yet received marketing authorization (Hofmann and Steinhilber, 2013). In fact, the discovery and evaluation of new FLAP-interfering chemotypes has been strongly limited by the fact that FLAP has no specific enzymatic functionality, which might be utilized as a measure for a simple and convenient compound screening approach. For this reason, experimental evidence for direct and functional FLAP targeting is limited and pharmacological evaluation of FLAP inhibitors has to rely on comparative analysis with previously recognized compounds (e.g. in terms of inhibition of 5-LOX product formation in cellular assays without direct 5-LOX inhibition), which has hampered the identification of novel clinically relevant compounds. In fact, research has mainly focused upon the structural optimization of the only two known chemotypes for FLAP inhibition, that is, the indole and quinoline cores (Sampson, 2009). Interestingly, the crystal structure of FLAP bound to two inhibitors has recently been elucidated and these studies have also provided functional insights into the role of FLAP in aiding 5-LOX for LTA$_4$ synthesis, by revealing the location of the inhibitor-binding site (Ferguson *et al.*, 2007; Ferguson, 2012). Although the resolution of the FLAP structure was too low for reliable structure-based design, we made use of the structural knowledge and combined it with ligand-based evidence to implement a rapid virtual screening strategy for the identification of new chemotypes for FLAP inhibitors (Banoglu *et al.*, 2012). Screening of a pre-compiled collection of 2.8 mio vendor compounds (153 mio conformations) using this three-dimensional pharmacophore query led to 1792 compounds matching the desired pharmacophoric features, and after filtering according to forcefield refinement and rescoring function, 192 virtual hits remained. Then by use of protein–ligand interaction fingerprint application of MOE, BRP-7 was revealed as the overall hit, but its pharmacological profile remained unexplored.

BRP-7, a non-acidic benzimidazole derivative bearing the isobutylphenylethyl fingerprint of ibuprofen, is a novel structure that lacks typical pharmacophoric moieties of FLAP inhibitors, such as indole core, carboxylic acid or a quinoline residue (Evans *et al.*, 2008; Sampson, 2009). Our present data show that BRP-7 displays the typical profile of FLAP inhibitors and exhibits pharmacological properties, which support its further development, that is, *in vivo* efficacy. Thus, similar to other FLAP inhibitors that typically fail to inhibit soluble 5-LOX in cell-free assays (Miller *et al.*, 1990; Hatzelmann *et al.*, 1993), BRP-7 failed to inhibit the activity of 5-LOX in cell homogenates (neutrophils and monocytes) or to inhibit isolated human recombinant 5-LOX at concentrations up to 100-fold higher than those needed to block cellular 5-LOX product synthesis by 50%. Similar features were observed for the FLAP inhibitor MK-866, whereas the direct 5-LOX inhibitor zileuton (Carter *et al.*, 1991) blocked 5-LOX activity in the cell-free assay equally well as in intact neutrophils, as expected.

A strong loss of potency in cell-free assays also holds true for direct 5-LOX inhibitors of the non-redox type, because of the lack of a reducing environment (Werz *et al.*, 1998; Fischer *et al.*, 2004), and this may also apply to BRP-7. However, unlike the non-redox-type 5-LOX inhibitors (Werz *et al.*, 1998; Fischer *et al.*, 2004), supplementation of neutrophil homogenates with GSH did not confer 5-LOX inhibitory properties to BRP-7, implying that the failure of BRP-7 to inhibit 5-LOX in cell-free assays is not due to a lack of reducing environment, but rather suggesting that BRP-7 acts on FLAP.

As FLAP may confer high 5-LOX-mediated product synthesis by increasing the utilization of endogenously released AA by 5-LOX (Abramovitz *et al.*, 1993; Ferguson, 2012), exogenous addition of AA as a substrate to some extent circumvents the requirement of FLAP. Accordingly, the potency of BRP-7 in the presence of exogenously added AA was reduced and no reduction of AA release was observed, again suggesting that BRP-7 interfers with FLAP. Finally, FLAP bound to the BRP-7-affinity matrix **1b** more than the control matrix **2b** carrying an inactive analogue, and this was reduced after competition with free BRP-7. Note that as compared to other FLAP inhibitors such as MK-866, BRP-7 showed a certain degree of selectivity with respect to other AA metabolizing enzymes (e.g. COX, 12/15-LOX) and MAPEG (e.g. mPGES-1). These data confirm the inhibition of AA transfer to 5-LOX as a primary effect of FLAP-interference, as suggested by previous studies (Ferguson *et al.*, 2007). Interestingly, these effects on 5-LOX translocation and 5-LOX/FLAP localization were only observed at higher concentrations than those required for inhibition of LTs, suggesting that BRP-7's direct effects on the interaction between 5-LOX and FLAP may be a secondary effect. It is notable that BRP-7 showed a higher potency in monocytes than in neutrophils, which may relate to differences in the role of FLAP or variations in the accessibility of the compound to the nuclear membrane between the different cell types.

BRP-7 suppressed 5-LOX product formation in HWB and was effective *in vivo* in reducing LT levels and the inflammatory reaction, as observed in two well-recognized models of acute inflammation (i.e. carrageenan-induced pleurisy in rats and zymosan-induced peritonitis in mice). This is in contrast to several 5-LOX inhibitors, which are often found to be inactive under these conditions (Pergola and Werz, 2010). Although the actual potency of BRP-7 appears slightly lower as compared to optimized indole- or quinoline-containing derivatives, the chemical nature of BRP-7 and its synthetic accessibility offer several possibilities for lead optimization. Importantly, we did not observe significant inhibition of CYP3A4 by BRP-7, whereas CYP inhibition has been a critical issue for certain FLAP inhibitors (Hutchinson *et al.*, 2009). Indeed, BRP-7 was also inactive on hERG and did not show significant cellular toxicity or obvious toxic effects in mice and rats. Together, we propose BRP-7 as novel chemotype that

inhibits LT biosynthesis by targeting FLAP, without affecting related enzymes, such as 5-LOX, 12/15-LOX, COX-1, COX-2 and mPGES-1, but with pharmacological relevance *in vivo* and potential for further development as a lead compound.

Acknowledgements

We gratefully acknowledge the financial support by The Scientific and Technological Research Council of Turkey (TÜBİTAK Grant No. 112S596). We thank Julia Seegers, Katrin Fischer, Petra Wiecha and Monika Listing for expert technical assistance.

Conflict of interest

None.

References

Abramovitz M, Wong E, Cox ME, Richardson CD, Li C, Vickers PJ (1993). 5-Lipoxygenase-activating protein stimulates the utilization of arachidonic acid by 5-lipoxygenase. Eur J Biochem 215: 105–111.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M, Peters JA, Harmar AJ and CGTP Collaborators (2013). The Concise Guide to PHARMACOLOGY 2013/14: Enzymes. Br J Pharmacol 170: 1797–1867.

Back M (2009). Inhibitors of the 5-lipoxygenase pathway in atherosclerosis. Curr Pharm Des 15: 3116–3132.

Bain G, King CD, Schaab K, Rewolinski M, Norris V, Ambery C *et al.* (2013). Pharmacodynamics, pharmacokinetics and safety of GSK2190915, a novel oral anti-inflammatory 5-lipoxygenase-activating protein inhibitor. Br J Clin Pharmacol 75: 779–790.

Bair AM, Turman MV, Vaine CA, Panettieri RA Jr, Soberman RJ (2012). The nuclear membrane leukotriene synthetic complex is a signal integrator and transducer. Mol Biol Cell 23: 4456–4464.

Banoglu E, Caliskan B, Luderer S, Eren G, Ozkan Y, Altenhofen W *et al.* (2012). Identification of novel benzimidazole derivatives as inhibitors of leukotriene biosynthesis by virtual screening targeting 5-lipoxygenase-activating protein (FLAP). Bioorg Med Chem 20: 3728–3741.

Byrum RS, Goulet JL, Griffiths RJ, Koller BH (1997). Role of the 5-lipoxygenase-activating protein (FLAP) in murine acute inflammatory responses. J Exp Med 185: 1065–1075.

Carter GW, Young PR, Albert DH, Bouska J, Dyer R, Bell RL *et al.* (1991). 5-Lipoxygenase inhibitory activity of zileuton. J Pharmacol Exp Ther 256: 929–937.

Claveau D, Sirinyan M, Guay J, Gordon R, Chan CC, Bureau Y *et al.* (2003). Microsomal prostaglandin E synthase-1 is a major terminal synthase that is selectively up-regulated during cyclooxygenase-2-dependent prostaglandin E2 production in the rat adjuvant-induced arthritis model. J Immunol 170: 4738–4744.

Evans JF, Ferguson AD, Mosley RT, Hutchinson JH (2008). What's all the FLAP about? 5-Lipoxygenase-activating protein inhibitors for inflammatory diseases. Trends Pharmacol Sci 29: 72–78.

Ferguson AD (2012). Structure-based drug design on membrane protein targets: human integral membrane protein 5-lipoxygenase-activating protein. Methods Mol Biol 841: 267–290.

Ferguson AD, McKeever BM, Xu S, Wisniewski D, Miller DK, Yamin TT *et al.* (2007). Crystal structure of inhibitor-bound human 5-lipoxygenase-activating protein. Science 317 (5837): 510–512.

Fischer L, Steinhilber D, Werz O (2004). Molecular pharmacological profile of the nonredox-type 5-lipoxygenase inhibitor CJ-13,610. Br J Pharmacol 142: 861–868.

Fischer L, Poeckel D, Buerkert E, Steinhilber D, Werz O (2005). Inhibitors of actin polymerisation stimulate arachidonic acid release and 5-lipoxygenase activation by upregulation of Ca^{2+} mobilisation in polymorphonuclear leukocytes involving Src family kinases. Biochim Biophys Acta 1736: 109–119.

Fischer L, Hornig M, Pergola C, Meindl N, Franke L, Tanrikulu Y *et al.* (2007). The molecular mechanism of the inhibition by licofelone of the biosynthesis of 5-lipoxygenase products. Br J Pharmacol 152: 471–480.

Follows RM, Snowise NG, Ho SY, Ambery CL, Smart K, McQuade BA (2013). Efficacy, safety and tolerability of GSK2190915, a 5-lipoxygenase activating protein inhibitor, in adults and adolescents with persistent asthma: a randomised dose-ranging study. Respir Res 14: 54.

Hatzelmann A, Fruchtmann R, Mohrs KH, Raddatz S, Muller-Peddinghaus R (1993). Mode of action of the new selective leukotriene synthesis inhibitor BAY X 1005 ((R)-2-[4-(quinolin-2-yl-methoxy)phenyl]-2-cyclopentyl acetic acid) and structurally related compounds. Biochem Pharmacol 45: 101–111.

Hofmann B, Steinhilber D (2013). 5-Lipoxygenase inhibitors: a review of recent patents (2010–2012). Expert Opin Ther Pat 23: 895–909.

Hutchinson JH, Li Y, Arruda JM, Baccei C, Bain G, Chapman C *et al.* (2009). 5-Lipoxygenase-activating protein inhibitors: development of 3-[3-*tert*-butylsulfanyl-1-[4-(6-methoxy-pyridin-3-yl)-benzyl]-5-(pyridin-2-ylmethoxy)-1*H*-indol-2-yl]-2,2-dimethyl-propionic acid (AM103). J Med Chem 52: 5803–5815.

Kent SE, Boyce M, Diamant Z, Singh D, O'Connor BJ, Saggu PS *et al.* (2013). The 5-lipoxygenase-activating protein inhibitor, GSK2190915, attenuates the early and late responses to inhaled allergen in mild asthma. Clin Exp Allergy 43: 177–186.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). Animal research: reporting *in vivo* experiments: the ARRIVE guidelines. Br J Pharmacol 160: 1577–1579.

Koeberle A, Siemoneit U, Buhring U, Northoff H, Laufer S, Albrecht W *et al.* (2008). Licofelone suppresses prostaglandin E2 formation by interference with the inducible microsomal prostaglandin E2 synthase-1. J Pharmacol Exp Ther 326: 975–982.

Koeberle A, Siemoneit U, Northoff H, Hofmann B, Schneider G, Werz O (2009). MK-886, an inhibitor of the 5-lipoxygenase-activating protein, inhibits cyclooxygenase-1 activity and suppresses platelet aggregation. Eur J Pharmacol 608: 84–90.

Kolaczkowska E, Shahzidi S, Seljelid R, van Rooijen N, Plytycz B (2002). Early vascular permeability in murine experimental peritonitis is co-mediated by resident peritoneal macrophages and mast cells: crucial involvement of macrophage-derived cysteinyl-leukotrienes. Inflammation 26: 61–71.

Laufer S, Tries S, Augustin J, Dannhardt G (1994a). Pharmacological profile of a new pyrrolizine derivative inhibiting the enzymes cyclo-oxygenase and 5-lipoxygenase. Arzneimittelforschung 44: 629–636.

Laufer SA, Augustin J, Dannhardt G, Kiefer W (1994b). (6,7-Diaryldihydropyrrolizin-5-yl)acetic acids, a novel class of potent dual inhibitors of both cyclooxygenase and 5-lipoxygenase. J Med Chem 37: 1894–1897.

McGrath JC, Drummond GB, McLachlan EM, Kilkenny C, Wainwright CL (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

Miller DK, Gillard JW, Vickers PJ, Sadowski S, Leveille C, Mancini JA *et al.* (1990). Identification and isolation of a membrane protein necessary for leukotriene production. Nature 343(6255): 278–281.

Pergola C, Werz O (2010). 5-Lipoxygenase inhibitors: a review of recent developments and patents. Expert Opin Ther Pat 20: 355–375.

Pergola C, Dodt G, Rossi A, Neunhoeffer E, Lawrenz B, Northoff H *et al.* (2008). ERK-mediated regulation of leukotriene biosynthesis by androgens: a molecular basis for gender differences in inflammation and asthma. Proc Natl Acad Sci U S A 105: 19881–19886.

Pergola C, Rogge A, Dodt G, Northoff H, Weinigel C, Barz D *et al.* (2011). Testosterone suppresses phospholipase D, causing sex differences in leukotriene biosynthesis in human monocytes. FASEB J 25: 3377–3387.

Pergola C, Jazzar B, Rossi A, Northoff H, Hamburger M, Sautebin L *et al.* (2012). On the inhibition of 5-lipoxygenase product formation by tryptanthrin: mechanistic studies and efficacy *in vivo*. Br J Pharmacol 165: 765–776.

Peters-Golden M, Henderson WR Jr (2007). Leukotrienes. N Engl J Med 357: 1841–1854.

Radmark O, Werz O, Steinhilber D, Samuelsson B (2007). 5-Lipoxygenase: regulation of expression and enzyme activity. Trends Biochem Sci 32: 332–341.

Raynauld JP, Martel-Pelletier J, Bias P, Laufer S, Haraoui B, Choquette D *et al.* (2009). Protective effects of licofelone, a 5-lipoxygenase and cyclo-oxygenase inhibitor, versus naproxen on cartilage loss in knee osteoarthritis: a first multicentre clinical trial using quantitative MRI. Ann Rheum Dis 68: 938–947.

Riccioni G, Back M (2012). Leukotrienes as modifiers of preclinical atherosclerosis? ScientificWorldJournal 2012: 490968.

Sampson AP (2009). FLAP inhibitors for the treatment of inflammatory diseases. Curr Opin Investig Drugs 10: 1163–1172.

Sardella R, Levent S, Ianni F, Caliskan B, Gerstmeier J, Pergola C *et al.* (2013). Chromatographic separation and biological evaluation of benzimidazole derivative enantiomers as inhibitors of leukotriene biosynthesis. J Pharm Biomed Anal 89C: 88–92.

Snowise NG, Clements D, Ho SY, Follows RM (2013). Addition of a 5-lipoxygenase-activating protein inhibitor to an inhaled corticosteroid (ICS) or an ICS/long-acting beta-2-agonist combination in subjects with asthma. Curr Med Res Opin 29: 1663–1674.

Stock NS, Bain G, Zunic J, Li Y, Ziff J, Roppe J *et al.* (2011). 5-Lipoxygenase-activating protein (FLAP) inhibitors. Part 4: development of 3-[3-tert-butylsulfanyl-1-[4-(6-ethoxypyridin-3-yl) benzyl]-5-(5-methylpyridin-2-ylmethoxy)-1*H*-indol-2-yl]-2,2-dimethylpropionic acid (AM803), a potent, oral, once daily FLAP inhibitor. J Med Chem 54: 8013–8029.

Surette ME, Palmantier R, Gosselin J, Borgeat P (1993). Lipopolysaccharides prime whole human blood and isolated neutrophils for the increased synthesis of 5-lipoxygenase products by enhancing arachidonic acid availability: involvement of the CD14 antigen. J Exp Med 178: 1347–1355.

Tretiakova I, Blaesius D, Maxia L, Wesselborg S, Schulze-Osthoff K, Cinatl J Jr *et al.* (2008). Myrtucommulone from *Myrtus communis* induces apoptosis in cancer cells via the mitochondrial pathway involving caspase-9. Apoptosis 13: 119–131.

Uozumi N, Kume K, Nagase T, Nakatani N, Ishii S, Tashiro F *et al.* (1997). Role of cytosolic phospholipase A2 in allergic response and parturition. Nature 390 (6660): 618–622.

Werz O, Szellas D, Henseler M, Steinhilber D (1998). Nonredox 5-lipoxygenase inhibitors require glutathione peroxidase for efficient inhibition of 5-lipoxygenase activity. Mol Pharmacol 54: 445–451.

Werz O, Klemm J, Samuelsson B, Radmark O (2001). Phorbol ester up-regulates capacities for nuclear translocation and phosphorylation of 5-lipoxygenase in Mono Mac 6 cells and human polymorphonuclear leukocytes. Blood 97: 2487–2495.

Werz O, Burkert E, Samuelsson B, Radmark O, Steinhilber D (2002). Activation of 5-lipoxygenase by cell stress is calcium independent in human polymorphonuclear leukocytes. Blood 99: 1044–1052.

Yao X, Anderson DL, Ross SA, Lang DG, Desai BZ, Cooper DC *et al.* (2008). Predicting QT prolongation in humans during early drug development using hERG inhibition and an anaesthetized guinea-pig model. Br J Pharmacol 154: 1446–1456.

Zarini S, Gijon MA, Folco G, Murphy RC (2006). Effect of arachidonic acid reacylation on leukotriene biosynthesis in human neutrophils stimulated with granulocyte-macrophage colony-stimulating factor and formyl-methionyl-leucyl-phenylalanine. J Biol Chem 281: 10134–10142.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.12625

Appendix S1 Supporting methods.

3.2 Manuscript II

An experimental cell-based model for studying the cell biology and molecular pharmacology of 5-lipoxygenase-activating protein in leukotriene biosynthesis

Gerstmeier, J., C. Weinigel, D. Barz, O. Werz and U. Garscha (2014)

Biochimica et Biophysica Acta 1840(9): 2961-2969.

In this study, a smart cell-based model stably expressing 5-LO alone or together with FLAP in HEK293 cells was established to (I) characterize the 5-LO/FLAP interaction (II) and to screen for putative LT biosynthesis inhibitors. Co-expression of FLAP significantly increased LT biosynthesis by promoting the formation of LTA_4. While FLAP inhibitors were only effective in intact cells co-expressing FLAP, direct 5-LO inhibitors did not distinguish between the two cell lines, and equally suppressed 5-LO product formation. All FLAP-mediated actions were abolished in cell homogenates, highlighting FLAP`s undisputed function for cellular LT biosynthesis. We conclude that FLAP stabilizes 5-LO at the nuclear membrane, and functions as a membrane anchor to promote efficient LTA_4 biosynthesis at this locale. Together, we provide an innovative and powerful mammalian expression system to investigate the 5-LO/FLAP interaction and to evaluate novel FLAP antagonists.

Contribution (80%): Cloning of FLAP sequence into mammalian expression plasmid, stable transfection of HEK293 cells with pcDNA3.1/neom(+)_5-LO and pcDNA3.1/hygro(-)_FLAP, purification of human leukocytes, 5-LO activity assays and inhibitor studies in HEK293 cells, monocytes, and neutrophils, HPLC analysis of 5-LO products, immunofluorescence microscopy, SDS-page and Western blot, [^{3}H]-AA release, analysis of data and preparation of graphs, analysis of statistics, writing the manuscript.

Biochimica et Biophysica Acta 1840 (2014) 2961–2969

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

An experimental cell-based model for studying the cell biology and molecular pharmacology of 5-lipoxygenase-activating protein in leukotriene biosynthesis

Jana Gerstmeier [a], Christina Weinigel [b], Dagmar Barz [b], Oliver Werz [a,*], Ulrike Garscha [a]

[a] Chair of Pharmaceutical/Medicinal Chemistry, Institute of Pharmacy, Friedrich-Schiller-University, Germany
[b] Institute of Transfusion Medicine, Jena University Hospital, 07743 Jena, Germany

ARTICLE INFO

Article history:
Received 26 March 2014
Received in revised form 9 May 2014
Accepted 25 May 2014
Available online 4 June 2014

Keywords:
5-Lipoxygenase
5-Lipoxygenase-activating protein
HEK293 cells
MK886
Arachidonic acid
12-Lipoxygenase

ABSTRACT

Background: Subcellular distribution of 5-lipoxygenase (5-LO) to the perinuclear region and interaction with the 5-LO-activating protein (FLAP) are assumed as key steps in leukotriene biosynthesis and are prone to FLAP antagonists.
Methods: FLAP and/or 5-LO were stably expressed in HEK293 cells, 5-LO products were analyzed by HPLC, and 5-LO and FLAP subcellular localization was visualized by immunofluorescence microscopy.
Results: 5-LO and FLAP were stably expressed in HEK293 cells, and upon Ca^{2+}-ionophore A23187 stimulation exogenous AA was efficiently transformed into the 5-LO products 5-hydro(pero)xyeicosatetraenoic acid (5-H(p)ETE) and the trans-isomers of LTB_4. A23187 stimulation caused 5-LO accumulation at the nuclear membrane only when FLAP was co-expressed. Unexpectedly, A23187 stimulation of HEK cells expressing 5-LO and FLAP without exogenous AA failed in 5-LO product synthesis. HEK cells liberated AA in response to A23187, and transfected HEK cells expressing 12-LO generated 12-HETE after A23187 challenge from endogenous AA. FLAP co-expression increased 5-LO product formation in A23187-stimulated cells at low AA concentrations. Only in cells expressing FLAP and 5-LO, the FLAP antagonist MK886 blocked FLAP-mediated increase in 5-LO product formation, and prevented 5-LO nuclear membrane translocation and co-localization with FLAP.
Conclusion: The cellular biosynthesis of 5-LO products from endogenously derived substrate requires not only functional 5-LO/FLAP co-localization but also additional prerequisites which are dispensable when exogenous AA is supplied; identification of these determinants is challenging.
General significance: We present a cell model to study the role of FLAP as 5-LO interacting protein in LT biosynthesis in intact cells and for characterization of putative FLAP antagonists.

1. Introduction

Leukotrienes (LT) are potent lipid mediators that are released during many human pathologic processes such as acute and chronic inflammation, allergy, cancer, and cardiovascular diseases [1]. They derive from arachidonic acid (AA) released by cytosolic phospholipase A_2 ($cPLA_2$) from phospholipids within the nuclear membrane. 5-Lipoxygenase (5-LO), a non-heme dioxygenase, metabolizes AA by stereospecific hydrogen abstraction at C-7, followed by radical migration and antarafacial oxygen insertion at C-5, generating 5(*S*)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HpETE). In a second step of 5-LO catalysis, the enzyme abstracts hydrogen at C-10 of 5-HpETE with subsequent radical migration to C-6 and double bond rearrangement to a conjugated triene. The radical relocates, dehydrates, and forms the instable epoxide leukotriene A_4 (LTA_4) [2,3]. Subsequently, LTA_4 can be further converted by other lipoxygenases to lipoxins [4], by LTA_4 hydrolase (LTA_4H) to leukotriene B_4 (LTB_4) [5], or conjugated with reduced glutathione by leukotriene C_4 synthase (LTC_4S) to LTC_4 [6]. Additionally, LTA_4 is non-enzymatically degraded to trans-isomers of LTB_4.

In contrast to other lipoxygenases, 5-LO is activated and regulated after cell stimulation by diverse factors including Ca^{2+}, phosphorylations, and stabilization by binding to coactosin-like protein (CLP) [7,8]. Activated 5-LO in the cytosol or soluble compartment of the nucleus migrates to the perinuclear region, where AA is released by $cPLA_2$ and believed to be delivered to 5-LO by the membrane bound 5-lipoxygenase-activating protein (FLAP). As far as is known, 5-LO is the only lipoxygenase that requires FLAP, or any transmembrane-bound helper protein, for full activity in the cell. It has been shown that FLAP

Abbreviations: 5-LO, 5-lipoxygenase; 5-H(p)ETE, 5-hydro(pero)xy-6-trans 8,11,14-cis-eicosatetraenoic acid; 12-LO, 12-lipoxygenase; AA, arachidonic acid; $cPLA_2$, cytosolic phospholipase A_2; FLAP, 5-lipoxygenase-activating protein; HEK cells, human embryonic kidney cells; LT, leukotriene; LTB_4, leukotriene B_4

* Corresponding author.

http://dx.doi.org/10.1016/j.bbagen.2014.05.016
0304-4165/

binds AA, and inhibition by the FLAP antagonist MK886 blocks LT formation and prevents 5-LO translocation in intact activated human leukocytes [9–11]. FLAP belongs to the superfamily of membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) [12]. However, it differs from other members in lacking any enzymatic activity and the ability to conjugate glutathione. Despite intense investigations during the last two decades and its unquestioned importance for 5-LO activity [10], whether and how FLAP interacts with 5-LO and how FLAP facilitates the transport of AA from $cPLA_2$ to 5-LO are incompletely understood.

Since FLAP does not possess enzymatic activity and the 5-LO/FLAP functional interaction is limited to the cellular context, it is challenging to study the function of FLAP outside a cellular environment independent of 5-LO. So far, functional interference with FLAP by antagonists can essentially be assessed only by means of decreased 5-LO product formation in intact cells but not in cell-free preparations where FLAP is dispensable for 5-LO activity. Nevertheless, the affinity of putative FLAP antagonists towards FLAP can be analyzed by a radio ligand binding assays using human leukocyte membrane preparations as FLAP source [13]. However, this method requires competitive binding of the inhibitor and excludes allosteric inhibition of FLAP. Moreover, studies on recombinant purified FLAP have limited applicability, as the protein needs a bilayer surrounding to express full functionality.

Previous studies have utilized mammalian cell lines such as osteosarcoma cells for expression of 5-LO and FLAP in order to study the interaction and functional coupling for LT biosynthesis [9,14]. However, the cell lines used were transiently transfected with 5-LO or artificial 5-LO-GFP constructs were investigated [15] that eventually limited the reproducibility and biological relevance, respectively. In this study, we established a HEK cell model stably expressing 5-LO with or without FLAP that may allow for detailed analysis of the functional role of FLAP for 5-LO cell biology. Such a cellular model may constitute a robust experimental system for studying directed protein mutations in order to reveal the critical residues involved in the dynamic 5-LO/FLAP interaction. Finally, application of this HEK cell model may provide new possibilities to design and characterize novel 5-LO/FLAP inhibitors.

2. Experimental procedure

2.1. Materials

Phorbol-12-myristate-13-acetat (PMA) was from Applichem (Darmstadt, Germany) and Rotiszint® eco plus from Roth (Karlsruhe, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin, streptomycin, trypsin/EDTA, and geneticin were from PAA Laboratories (Coelbe, Germany). Lipofectamine LTX Reagent Plus, 10% non-immune goat serum, Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 555 goat anti-mouse, diamidino-2-phenylindol (DAPI), hygromycin B, pcDNA3.1/Hygro (−) vector kit, and chemically competent *Escherichia coli* cells (OneShot Top10) were from Invitrogen (Darmstadt, Germany). Phusion High fidelity polymerase, restriction enzymes, and GenJet-plasmid midiprep kit were from Fermentas (Darmstadt, Germany). The mouse anti-5-LO monoclonal antibody, the FLAP-cDNA-containing plasmid pSG5_FLAP, and the pcDNA3.1/neom (+)_5-LO-vector were generous gifts by Dr. Dieter Steinhilber (Goethe University Frankfurt, Germany). The rabbit anti-FLAP polyclonal antibody was from Abcam (Cambridge, UK) and the mouse anti-12-LO monoclonal antibody was from Santa Cruz. Tritium labeled [5,6,8,9,11,12,14,15-^{3}H]AA was from Biotrend Chemicals GmbH (Cologne, Germany). The FLAP inhibitor MK886 was from Cayman Chemicals (AnnArbor, US), zileuton (N-(1-benzo[b]thien-2-ylethyl)-N-hydroxyurea) was from Sequoia Research Products (Oxford, UK) and the $cPLA_2\alpha$ inhibitor RSC-3388 (N-{(2S,4R)-4-(biphenyl-2-ylmethyl-isobutyl-amino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl)-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl]acrylamide, HCl) as well as mowiol was from Calbiochem (Bad Soden, Germany). Oligonucleotides were from Tib Molbiol (Berlin, Germany). HPLC solvents were from Merck (Darmstadt, Germany). AA, Ca^{2+}-ionophore A23187, prostaglandin (PG)B_1, dNTPs, and all other chemicals were from Sigma-Aldrich (Deisenhofen, Germany) unless stated otherwise.

2.2. Cells

HEK293 cells were cultured as monolayers at 37 °C and 5% CO_2 in DMEM supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. HEK cell lines stably expressing 5-LO and/or FLAP were selected using 400 μg/ml geneticin and/or 200 μg/ml hygromycin B, respectively.

Human neutrophils and monocytes were freshly isolated from leukocyte concentrates obtained from the Institute of Transfusion Medicine, University Hospital Jena, as described [16]. In brief, neutrophils were isolated by dextran sedimentation, centrifuged on lymphocyte separation medium (LSM 1077, PAA, Coelbe, Germany) and subjected to hypertonic lysis of erythrocytes.

Monocytes were separated by adherence to culture flasks from peripheral blood mononuclear cells, as described [17]. Neutrophils and monocytes were resuspended in PBS plus glucose (0.1%) to a final cell density of 5×10^6 and 2×10^6 cells/ml, respectively.

2.3. Cloning of FLAP and platelet-type 12(S)-LO

Standard protocols for molecular biology were applied. The pSG5-FLAP vector was used as a template for amplification of the FLAP coding sequence, using primers extended by EcoRV and HindIII restriction sites at the 5′ and 3′ ends, respectively (forward 5′-gatatcatggatcaagaaactg, reverse 5′-gatgctattgctttatttgta). The cDNA was ligated between the EcoRV and HindIII cloning sites of pcDNA3.1/Hygro (−), generating the pcDNA3.1/Hygro (−)_FLAP expression vector. The cDNA of 12(S)-LO was amplified in two partly overlapping fragments of 1217 bp (nucleotides 1–1211) and 1337 bp (nucleotides 661–1989). A KpNI and XhoI restriction site was introduced in front of the ATG (5′ ggtaccATG) and after the stop codon (5′ ctcgagTCA) by PCR technology, respectively. The fragments were sequentially ligated (KpNI-EcoRI and EcoRI-XhoI) into the pcDNA3.1/Neom (+) vector, generating the pcDNA3.1/Neom(+)_12-LO expression vector.

2.4. Stable expression of 5-LO, FLAP and 12(S)-LO in HEK293

Transfection of HEK cells was performed using lipofectamine according to the instructions of the agent (Invitrogen, Darmstadt, Germany). Briefly, HEK cells were grown until ~60% confluency and 2 h prior transfection the medium was replaced by medium without antibiotics ("reduced medium"). Transfection mix composed of "reduced medium", 40 μg of purified pcDNA3.1/neom (+)_5-LO or pcDNA3.1/neom (+)_12-LO, and lipofectamine was added drop wise onto the cells. After 24 h, the medium was replaced by complete medium (with antibiotics) and cultured for additional 24 h. Then, cells expressing 5- or 12-LO were selected by 400 μg/ml geneticin. Stable transfectants were screened by activity tests and expression was verified by immunoblotting. Once a stable 5-LO-expressing cell line was generated, cells were co-transfected with a FLAP-coding construct (pcDNA3.1/Hygro (−) _FLAP) and selected using 200 μg/ml hygromycin B.

2.5. Determination of LO product formation in transfected HEK293 cells

In order to determine 5-LO product formation in intact cells, HEK cells expressing 5-LO +/− FLAP were harvested by trypsinization, centrifuged (1200 rpm; 10 min; 4 °C), and resuspended in PGC buffer (PBS/0.1% glucose/1 mM $CaCl_2$). Cells (1×10^6 /ml) pre-incubated with inhibitors or vehicle (0.1% DMSO) at 37 °C for 15 min and subsequently stimulated with 2.5 μM A23187 plus the indicated concentrations of AA at 37 °C. The reaction was stopped after 10 min by

addition of 1 ml ice cold methanol. The internal standard (200 ng PGB_1) and acidified PBS were added and subjected to solid phase extraction on C18-columns (100 mg, UCT, Bristol, PA, USA). 5-LO metabolites were eluted by methanol and analyzed by RP-HPLC using C-18 Radial-PAK column (Waters, Eschborn, Germany), as described before [18]. 5-LO products include the all-*trans*-isomers of LTB_4 and 5-H(p)ETE.

For determination of 5-LO products in cell homogenates, cells were harvested as described above, resuspended in ice cold PBS containing 1 mM EDTA, and sonicated for 3 × 10 sec, at 4 °C. Samples (corresponding to 1×10^6 cells/ml) were pre-incubated with inhibitors or vehicle (0.1% DMSO) at 4 °C for 15 min before addition of 2 mM $CaCl_2$ and indicated concentrations of AA for 10 min at 37 °C. 5-LO products were extracted and analyzed as described for intact cells. To determine 12-LO activity in intact cells, HEK cells stably expressing 12-LO were harvested as described above and stimulated with 2.5 μM A23187 at 37 °C with or without exogenous AA, as indicated. The reaction was stopped at the indicated time points by addition of 1 ml methanol and 12(*S*)-hydro(pero)xy-5,8-cis-10-trans-14-cis-eicosatetraenoic acid (12(*S*)-H(p)ETE) was analyzed by RP-HPLC, as described above.

2.6. Determination of LO product formation in neutrophils and monocytes

Intact neutrophils or monocytes were resuspended in 1 ml PGC buffer and stimulated with 2.5 μM A23187 with or without the indicated concentrations of AA for 10 min at 37 °C. The reaction was stopped with 1 ml methanol on ice and acidified PBS as well as the internal standard (200 ng PGB_1) was added to each sample. LO metabolites were isolated by solid phase extraction and analyzed by RP-HPLC, as described above.

2.7. Analysis of subcellular localization by immunofluorescence microscopy

HEK cells expressing 5-LO +/− FLAP were seeded onto acid-washed (50% sulfuric acid) and poly-L-lysine (0.01%)-coated glass coverslips and cultured for 48 h at 37 °C until ~60% confluency. Cells were pre-incubated with test compounds or vehicle (0.1% DMSO) for 10 min before stimulation with 2.5 μM A23187 for 5 min at 37 °C. The cells were fixed with 4% paraformaldehyde solution, followed by addition of 50 mM ammonium chloride to reduce fixative-induced autofluorescence. Acetone (3 min, 4 °C) was used to permeabilize the cells, before blocking with 10% non-immune goat serum. The cells were incubated for 1 h with mouse monoclonal anti-5-LO antibody (1:100) and rabbit polyclonal anti-FLAP antibody (5 μg/ml). The coverslips were intensively washed with PBS before staining with the fluorophore-labeled secondary antibodies Alexa Fluor 488 goat anti-rabbit (1:500) and Alexa Fluor 555 goat anti-mouse (1:500) for 20 min in the dark. DNA was stained with 0.1 μg/ml DAPI. The coverslips were mounted on glass slides with mowiol containing 2.5% n-propyl gallate. The cells were visualized by a Zeiss Axiovert 200 M microscope, and a Plan Neofluar × 100/1.30 Oil (DIC III) objective (Carl Zeiss, Jena, Germany). An AxioCam MR camera (Carl Zeiss) was used for image acquisition.

2.8. Aracidonic acid release

Cells (2.5×10^6 /ml) were resuspended in DMEM without additives and incubated with 0.5 μCi/ml ^{3}H-labelled AA (specific activity 200 Ci/mmol) for 2 h at 37 °C and 6% CO_2. Cells were washed twice with PBS containing 0.1% glucose and 0.2% fatty acid-free BSA. The cell number was adjusted to 1×10^6 /ml and 1 mM $CaCl_2$ was added to the incubation buffer (PBS/0.1% glucose/1 mM $CaCl_2$/0.2% fatty acid-free BSA). The cells were pre-incubated with 1 μM $cPLA_2\alpha$ inhibitor RSC-3388 or vehicle (0.1% DMSO) at 37 °C. After 15 min, vehicle (0.1% DMSO) or 0.1 μM PMA was added, incubated for additional 10 min at 37 °C, and subsequently stimulated with 5 μM A23187 for 30 min at 37 °C. Samples were placed on ice (10 min) and centrifuged (500 ×*g*, 10 min, and 4 °C). Aliquots (300 μl) of the supernatants were combined with 2 ml Rotiszint® eco plus and assayed for radioactivity by scintillation counting (Micro Beta Trilux, Perkin Elmer, Waltham, MA).

2.9. SDS-page and Western blot analysis

Cell lysates, corresponding 1×10^6 cells, were separated on 10% and 16% polyacrylamide gels, respectively, and blotted onto nitrocellulose membranes (Hybond ECL, GE Healthcare, Freiburg, Germany), as described [19]. The membranes were incubated with primary antibodies (mouse anti-5-LO, 1:1000; rabbit anti-FLAP, 0.1 μg/ml and mouse anti-12-LO, 1:1000) with subsequent detection using IRDye 800CW-labeled anti-rabbit and/or anti-mouse antibodies (1:10,000 each). Immunoreactive bands were visualized by an Odyssey infrared imager (LI-COR Biosciences).

2.10. Statistics

Results are expressed as means ± standard error of the mean (SEM) of *n* observations, where *n* represents the number of experiments performed at different days in duplicates unless stated otherwise. The IC_{50} values were determined by interpolation on semi-logarithmic graphs with GraphPad Prism 4 and validated with GraphPad InStat (Graphpad Software Inc., San Diego, CA). Statistical evaluation of the data was performed by one-way ANOVA followed by a Bonferroni *post hoc* test for multiple comparisons. Where appropriate, Student's *t* test was applied. A *P*-value of <0.05 (*) was considered significant.

3. Results

3.1. 5-LO product formation in HEK293 cells stably expressing 5-LO with or without FLAP

HEK cells were transfected with cDNA of either 5-LO (HEK-5-LO), 5-LO and FLAP (HEK-5-LO/FLAP) or 12-LO (HEK-12-LO). Colonies that stably express LOs and FLAP were selected by geneticin and hygromycin B, respectively. Expression of 5-LO, 12-LO and FLAP at the protein level in the respected cells was verified by immunoblotting (Fig. 1A,B). To our surprise, activation of HEK-5-LO/FLAP and HEK-5-LO with A23187 caused no significant formation of the 5-LO products (<2 ng/10^6 cells), while control incubations with A23187-stimulated human neutrophils or monocytes caused substantial 5-LO product synthesis under the same incubation conditions (Fig. 1C), as reported before [17,20]. In contrast, HEK cells transfected with 12-LO produced 12-H(p)ETE as AA-derived 12-LO metabolite upon A23187 stimulation (Fig. 1D). Note that 12-H(p)ETE formation was continuously formed for more than 15 min after a lag phase of around 2 min (Fig. 1E), which was surprising since it has been shown that platelet-type 12-LO metabolizes endogenously released AA extremely fast (within seconds) after A23187 stimulation [21].

Interestingly, upon addition of 3 or 10 μM AA, A23187-activated HEK-5-LO/FLAP and HEK-5-LO cells produced massive and even superior amounts of 5-LO products (5-H(p)ETE and trans-isomers of LTB_4) as compared to neutrophils and monocytes treated the same way (Fig. 1C). Because exogenous AA was necessary for cellular 5-LO product synthesis in A23187-activated HEK-5-LO and HEK-5-LO/FLAP cells, it appeared possible that these cells fail to provide sufficient AA as substrate for 5-LO/FLAP from endogenous sources, i.e., $cPLA_2$-mediated hydrolysis of membrane phospholipids. However, a significant increase in AA release from ^{3}[H] AA-labelled HEK cells (regardless of transfection) was evident after A23187 stimulation, which could be blocked by the $cPLA_2$ inhibitor RSC-3388. Together, our data show that endogenously released AA in HEK cells expressing both 5-LO and FLAP cannot be converted to 5-LO products even though 12-LO transforms endogenous AA. However, exogenous AA is substantially converted by 5-LO in HEK cells with or without FLAP.

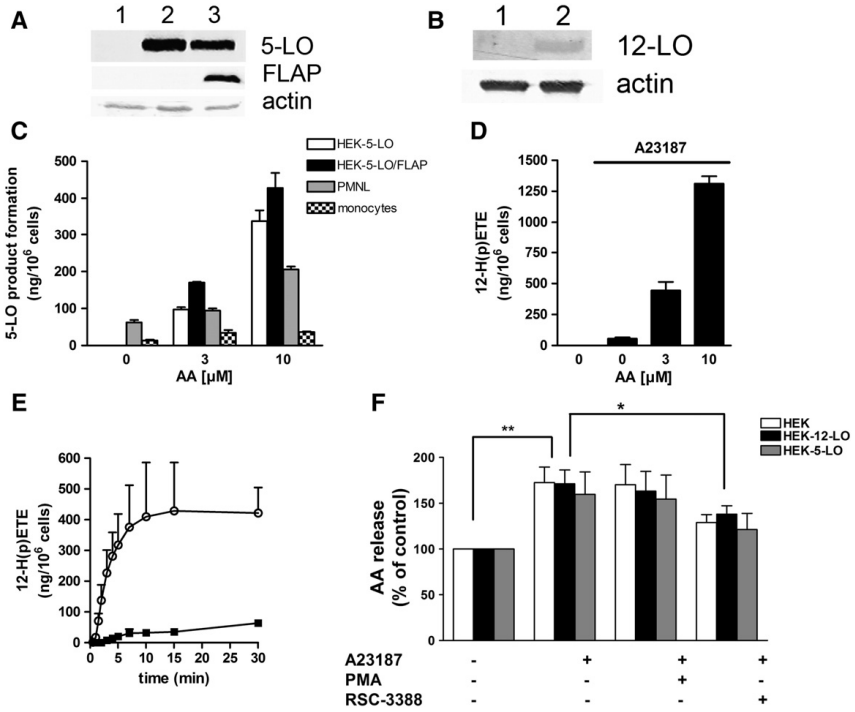


Fig. 1. Expression of 5-LO, FLAP, and 12-LO, and analysis of cellular eicosanoid biosynthesis in HEK293 cells. (A) Cell lysates (1×10^6 cells) of untransfected HEK cells (control) in lane 1, 5-LO-expressing HEK-5-LO in lane 2, and 5-LO/FLAP co-expressing HEK cells in lane 3. LOs and FLAP were resolved on 10% and 16% polyacrylamide gels, respectively. (B) Western blot analysis for 12-LO. Cell lysates of 12-LO-expressing HEK cells (lane 2) versus untransfected cells (lane 1). Images are representative for three independent blots and normalized against actin. (C) 5-LO product formation in intact HEK cells upon A23187 stimulation with or without AA, as indicated. HEK-5-LO, white; HEK-5-LO/FLAP, black; neutrophils, grey; monocytes, pattern. Data are given as means ± SEM, $n = 3$–5. (D) 12-H(p)ETE formation in cells stably expressing 12-LO with and without exogenous AA. (E) Time course of 12-LO product formation in intact HEK cells stably expressing 12-LO upon A23187 stimulation (-■-) and A23187 stimulation plus 3 μM exogenous AA (-○-). Data are representative for three comparable experiments. (F) AA release in untransfected HEK293 cells as well as in 5-LO and 12-LO expressing cells. Data are means ± SEM, $n = 3$–4, duplicates; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, vs unstimulated control or stimulated vs. inhibitor respectively, ANOVA plus Bonferroni.

Next, the concentration of exogenously added AA was varied. Addition of 3 μM AA together with A23187 revealed a significant higher product formation in HEK-5-LO/FLAP as compared to HEK-5-LO cells (Fig. 2A). However, this difference in 5-LO product formation was abolished at 10 or 30 μM of exogenous AA (Fig. 2A). Note that in homogenates of HEK-5-LO/FLAP and HEK-5-LO cells the formation of 5-LO product was not significantly different, irrespective of the AA concentration (i.e., 3, 10 or 30 μM AA) (Fig. 2B).

Of interest, the 5-LO product profile (5-H(p)ETE and trans-isomers of LTB_4) differed significantly between HEK-5-LO and HEK-5-LO/FLAP cells (Fig. 2C/E). Thus, A23187-stimulated HEK-5-LO/FLAP cells converted exogenous AA (at 3 μM AA) to the final product LTA_4 to a greater extent than HEK-5-LO cells. The ratio of 5-H(p)ETE versus trans-isomers of LTB_4 decreased from 6:1 to 2:1 when FLAP was co-expressed (Fig. 2C/E). However, in homogenates the 5-LO product profile is equal in cells with or without FLAP independent of the AA concentration (Fig. 2D/F), as expected. Together, the data suggest that the presence of FLAP stimulates 5-LO product formation at low substrate concentrations (i.e., at 3 μM AA) and shifts the ratio from 5-H(p)ETE towards LTA_4 metabolites in intact HEK cells, whereas in cell-free assays based on experiments with HEK cell homogenates FLAP is not operative. This indicates that FLAP preferentially promotes the 5-LO-mediated conversion of 5-HpETE to LTA_4 (i.e., the synthase activity of 5-LO).

3.2. MK886 differentially inhibits 5-LO product formation in HEK cells depending on the presence of FLAP

Inhibitor studies using the FLAP antagonist MK886 that suppresses cellular LT biosynthesis with IC_{50} values in the low nanomolar range [22,23], and the direct 5-LO inhibitor zileuton [24] were performed in the described transfected HEK cell lines. When HEK-5-LO/FLAP cells were stimulated with A23187 plus 3 μM AA, MK886 reduced 5-LO product formation by ~35% at 30 nM. Higher concentrations of MK886 up to 3 μM did not further suppress 5-LO product synthesis leading to a plateau of the curve (Fig. 3A). At rather high concentrations of MK886 (i.e., 10 μM) 5-LO product formation was further impaired (by ~80%). As expected, in HEK-5-LO cells MK886 at nanomolar concentrations failed to inhibit 5-LO product formation, and only at high concentrations (3 and 10 μM) a similar reduction was observed as for HEK-5-LO/FLAP cells (Fig. 3A). Note that the elevated 5-LO product formation in HEK-5-LO/FLAP versus HEK-5-LO cells stimulated by A23187 plus 3 μM AA was reduced by 30 nM MK886 down to the level of cells lacking FLAP (Fig. 3C). In contrast, 30 nM MK886 failed to diminish 5-LO product synthesis in HEK-5-LO cells that lack FLAP (Fig. 3C). In homogenates, MK886 was not able to reduce 5-LO product formation regardless of the presence of FLAP (Fig. 3B). In contrast to MK886, the 5-LO inhibitor zileuton reduced 5-LO product formation in HEK cells

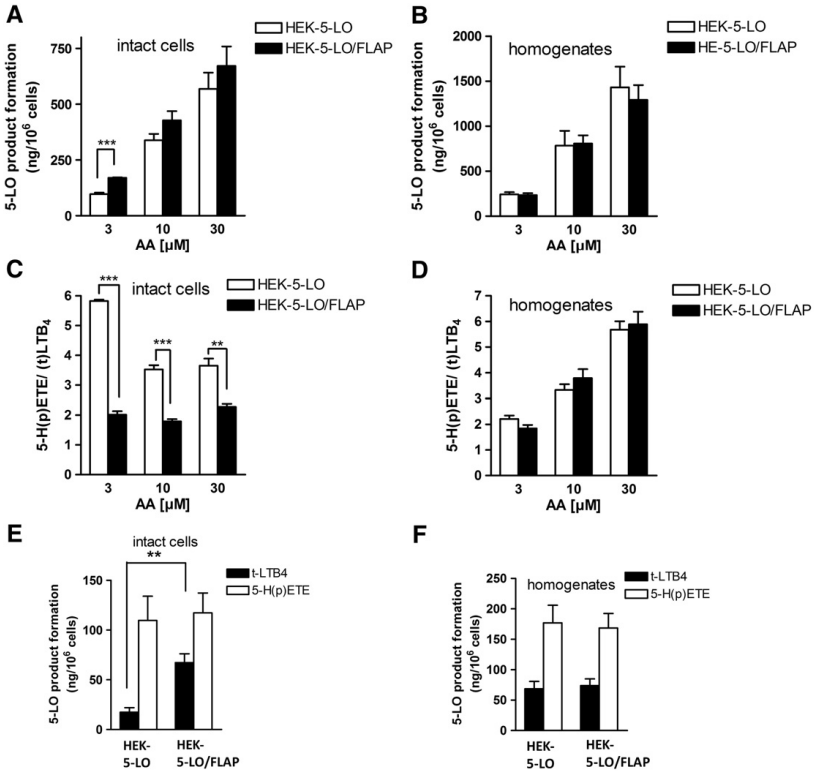


Fig. 2. 5-LO product synthesis in cell-based and cell-free assays using transfected HEK293 cells as source for 5-LO enzyme. Absolute amounts of 5-LO products in intact cells (A) and homogenates (B) after addition of different concentrations of exogenous AA. Cells were incubated with 2.5 μM A23187 and the indicated concentrations of AA. After 10 min at 37 °C, 5-LO products formed were analyzed by RP-HPLC. Ratio of 5-H(p)ETE to the trans-isomers of LTB_4 (trans-LTB_4) in intact cells (C) and homogenates (D). Formation of the trans-isomers of LTB_4 (trans-LTB_4) and 5-H(p)ETE by HEK-5-LO and HEK-5-LO/FLAP in (E) intact cells and (F) in cell homogenates. Data are expressed in means ± SEM; $n = 4$, duplicates; $^{***}p < 0.001$, $^{**}p < 0.01$; HEK-5-LO vs. HEK-5-LO/FLAP, Student's *t* test.

irrespective of FLAP expression with comparable potencies, in intact cells (Fig. 3E) and in homogenates (Fig. 3F) with IC_{50} values as described before for human leukocytes [10].

Since FLAP apparently promotes 5-LO-mediated conversion of 5-HpETE to LTA_4, we investigated whether MK886 may differentially suppress formation of LTA_4 metabolites (trans-isomers of LTB_4) versus 5-H(p)ETE. As shown in Fig. 3D, MK886 increased the ratio of 5-H(p)ETE/trans-isomers of LTB_4 from 2:1 to 2.6:1 at a concentration of 10 nM and 1 μM, respectively. In contrast, the 5-LO inhibitor zileuton did not change the ratio between 5H(p)ETE and the trans-isomers of LTB_4 (Fig. 3D). Therefore, inhibition of FLAP by MK886 seems to impair the second step in 5-LO catalysis, that is, the formation of LTA_4 from 5-HpETE.

3.3. FLAP is required for accumulation of 5-LO in the perinuclear region of HEK cells

It was proposed that in intact cells, translocation of 5-LO from the cytosol to the perinuclear region is necessary to access endogenously released AA by $cPLA_2$ from the nuclear membrane via FLAP [25]. Since 5-LO product formation in HEK-5-LO/FLAP could only be detected when exogenously AA was added, it appeared possible that 5-LO translocation and association with FLAP after A23187 stimulation are not properly operative. Analysis of 5-LO in HEK-5-LO cells by immunofluorescence microscopy revealed a rather homogenous (soluble) intranuclear staining in resting cells (Fig. 4A). Upon stimulation, 5-LO essentially remained within the nucleus but seemingly accumulated in certain intranuclear districts without marked association with the nuclear membrane. In sharp contrast, in HEK-5-LO/FLAP cells, intranuclear 5-LO translocated to the perinuclear region and clearly co-localized with FLAP (Fig. 4B). Next, we investigated if the FLAP antagonist MK886 blocks co-localization of 5-LO with FLAP. Indeed, MK886 (300 nM) prevented co-localization of 5-LO and FLAP, and 5-LO subcellular localization resembled the pattern of unstimulated HEK-5-LO/FLAP cells and/or the pattern of A23187-activated HEK-5-LO cells (Fig. 4B).

4. Discussion

We present here a cellular model using HEK293 cells that are stably transfected with 5-LO together or without FLAP for studying the functional interaction between the two proteins. Although 5-LO and FLAP are markedly expressed in HEK-5-LO/FLAP cells and stimulation with A23187 plus exogenous AA leads to substantial biosynthesis of 5-LO products comparable to neutrophils or monocytes, A23187 alone is insufficient to evoke 5-LO product synthesis in HEK-5-LO/FLAP cells. This is in sharp contrast to primary neutrophils and monocytes [26].

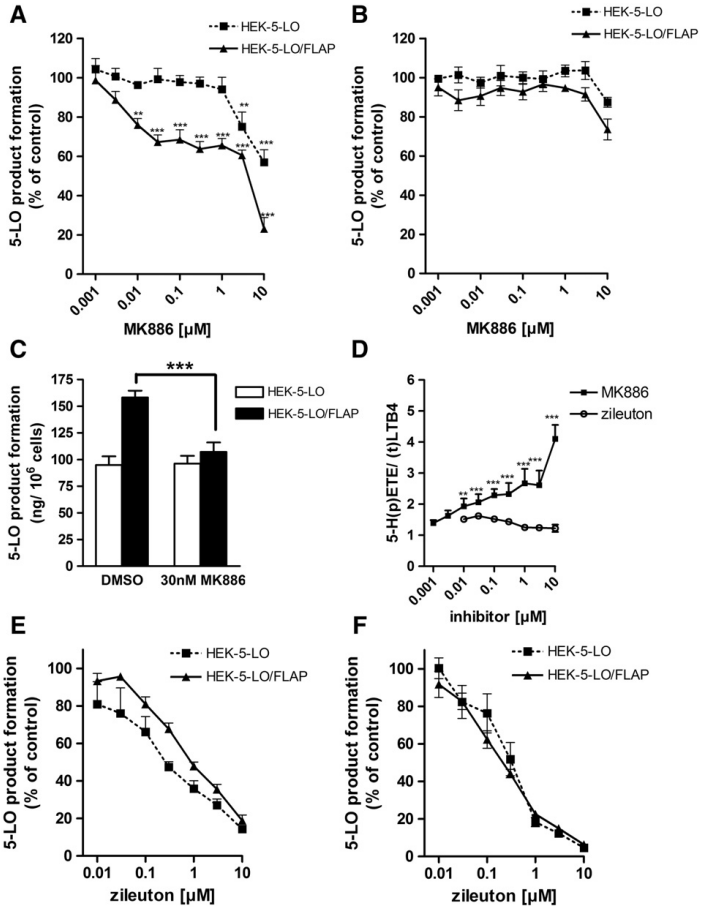


Fig. 3. MK886 inhibits 5-LO product formation in intact HEK293 cells expressing 5-LO and FLAP. (A) Intact HEK cells and (B) homogenates were preincubated with MK886 or vehicle (0.1% DMSO) for 15 min at 37 °C prior to stimulation with 2.5 μM A23187 plus 3 μM AA. After another 10 min at 37 °C 5-LO products formed were analyzed by RP-HPLC. (C) Leukotriene formation in HEK-5-LO and HEK-5-LO/FLAP cells preincubated with vehicle or 30 nM MK886 and stimulated by 2.5 μM A23187 and 3 μM AA. D) Ratios of 5-H(p)ETE to all trans-isomers of LTB_4 (trans-LTB_4) in HEK-5-LO/FLAP, incubated with increasing concentrations of MK886 (-■-) and zileuton (-○-). Intact cells (E) and homogenates (F) were preincubated for 15 min with zileuton, stimulated and analyzed as described above. Data are expressed in means ± SEM; $n = 3$–5, duplicates; $^{***}p < 0.001$, $^{**}p < 0.01$; inhibitor vs. vehicle (DMSO) control, ANOVA + Bonferroni test.

On the other hand, FLAP promoted 5-LO product formation in HEK cells in particular towards LTA_4 at moderate AA levels (i.e., 3 μM), and this FLAP-dependent increase in 5-LO product formation was efficiently blocked by the FLAP antagonist MK886 at nanomolar concentrations. Moreover, in HEK-5-LO/FLAP cells 5-LO clearly associated with FLAP at the perinuclear region upon A23187 challenge. Of interest, p12-LO was able to convert endogenously released AA, which demonstrates AA liberation in A23187 activated HEK cells. In conclusion, our study provides evidence for the existence of a more complex and fine-tuned relationship among 5-LO, FLAP and $cPLA_2$ in LT synthesis, which is operative in primary neutrophils and monocytes but seemingly cannot be completely recapitulated in transfected HEK cells.

Among the various LOs, the 5-LO exhibits extraordinary properties regarding activation, stability, and the essential nuclear membrane translocation process in order to reach FLAP upon stimulation [25]. FLAP is considered to be absolutely essential for LT biosynthesis *in vivo*, as either FLAP gene deletion or pharmacological interference with FLAP (e.g. by MK886) in mice completely abolished LT formation [27]. Also in human whole blood or isolated cells, FLAP inhibitors efficiently repress 5-LO product synthesis [28]. FLAP is distributed on both the inner and outer nuclear membranes and the existence of two pools of FLAP has been proposed [29]. Thus a complex of FLAP together with 5-LO and membrane-bound LTC_4S is formed in RBL-2H3 cells at the outer nuclear membrane that promotes the formation of cysLTs, whereas on the inner membrane (which is devoid of LTC_4S), the complex may consist only of 5-LO and FLAP in murine neutrophils and presumably interacts with nuclear soluble LTA_4H to produce LTB_4 [30]. FLAP can bind AA and certain other fatty acids [31,32], and may transport AA to 5-LO for efficient metabolism. In fact, formation of the 5-LO/FLAP complex in intact cells depends on the presence of AA [33]. Meanwhile, the structures of both 5-LO and FLAP are known [34,35]. However, even though there is substantial evidence for the necessity

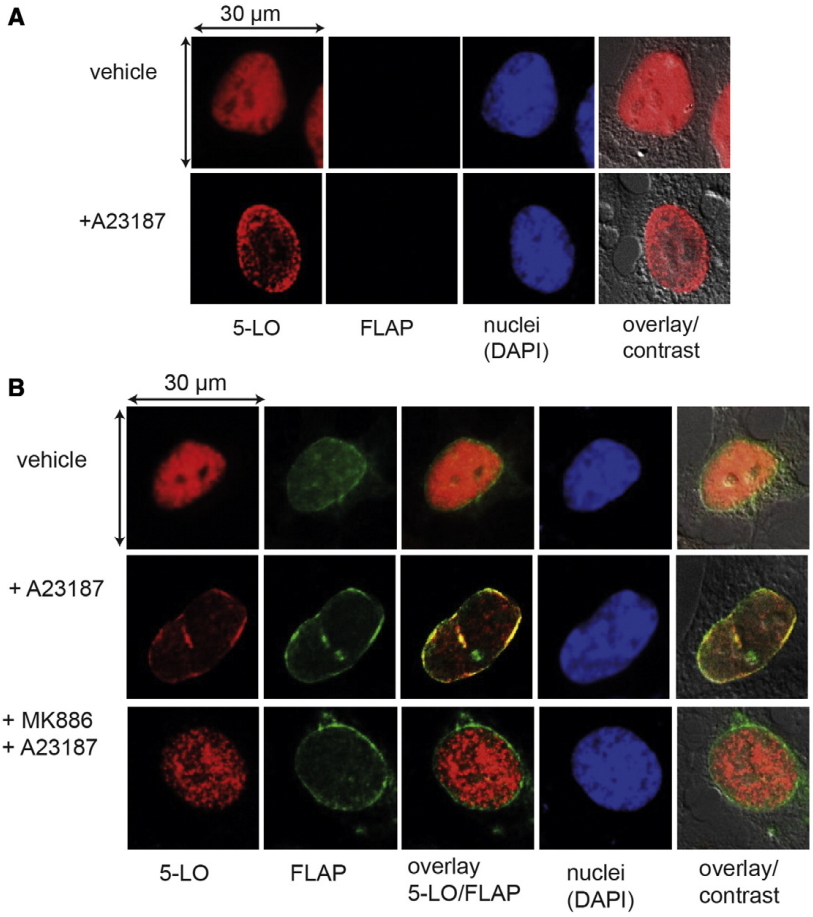


Fig. 4. Subcellular localization of 5-LO and FLAP in transfected HEK293 cells. Subcellular localization of 5-LO, analyzed by immunofluorescence microscopy, in resting cells and upon A23187 stimulation (2.5 µM; 5 min, 37 °C) is shown for cells expressing 5-LO (A) and 5-LO and FLAP (B). Cells co-expressing 5-LO and FLAP were preincubated with MK886 (300 nM, 10 min, 37 °C) or vehicle (0.1% DMSO) as indicated. Results are representative of three independent experiments. Images show single staining for 5-LO (Alexa Fluor 555, red), FLAP (Alexa Fluor 488, green), overlay of 5-LO and FLAP, DNA (DAPI, blue) and overlay with contrast; scale bar = 30 µm.

of FLAP for LT formation and for the establishment of 5-LO/FLAP complexes at various sites in the perinuclear region, how FLAP and 5-LO interact with each other and how FLAP stimulates 5-LO product synthesis remain elusive.

In this report, we developed an experimental cell model that may allow studying the functional interaction between 5-LO and FLAP to get deeper insights into the putative role of FLAP in the modulation of cellular 5-LO product formation. Thus, in contrast to previous models using primary cells or cell lines endogenously expressing FLAP and 5-LO [27], our cell-based model allows to investigate the consequences of site-directed mutation of 5-LO and/or FLAP in stably expressed in HEK cells. Reasonable candidate residues might be F177 and Y181 in 5-LO that appear to preclude AA entry into the 5-LO active site [34]. Indeed, substantial and reproducible amounts of FLAP and 5-LO protein were expressed in HEK-5-LO/FLAP cells, and AA given exogenously to intact A23187-activated cells or corresponding homogenates was effectively converted to the direct 5-LO products trans-isomers of LTB_4 and 5-H(p)ETE.

An unexpected major drawback in the course of our studies was, however, the failure of HEK-5-LO/FLAP cells to generate 5-LO products from endogenously released AA upon A23187 stimulation. HEK cells were shown to express $cPLA_2$ [36] and we confirmed AA release from A23187-activated HEK cells as well as transformation of endogenously liberated AA by 12-LO. These data exclude an absolute lack of free AA in HEK cells after stimulation with A23187. However, compared to human primary monocytes that respond with pronounced AA release upon A23187 stimulation (studied in parallel in our laboratory [37]), the liberation of AA from HEK cells is minute and rather delayed which might be insufficient to enable 5-LO product formation. Nevertheless, this moderate AA release in HEK293 cells seemingly affords generation of 12-H(p)ETE from endogenous substrate by stably transfected p12-LO. Note that only after exogenous addition of 3 µM AA, 5-LO

products were clearly detectable in HEK cells. Interestingly, comparison of 5-LO activity at low AA concentrations (3 μM) revealed significantly higher amounts of 5-LO products formed in cells expressing FLAP, indicating that FLAP was only operative under these conditions. In the HEK-cell model, at high concentrations of AA (10 μM and 30 μM) the FLAP-mediated increase in 5-LO product formation was not significant. This coincides with the reduced potency of FLAP inhibitors in intact human leukocytes with increased exogenous AA [38,39]. The functionality of FLAP is also supported by the fact that HEK-5-LO cells metabolized AA essentially to 5-H(p)ETE whereas co-expression with FLAP promoted the synthase activity of 5-LO with an increased formation of LTA_4 reflected by a higher level of trans-isomers of LTB_4. These data are consistent with observations in leukocytes and transfected osteosarcoma cells [40]. Note that under conditions where FLAP is dispensable for 5-LO product synthesis, as for example in cell homogenates or when excess of substrate (10 or 30 μM AA) is presented, the ratio between 5-H(p)ETE and LTA_4 metabolites were the same for cells with or without FLAP.

The role of FLAP for 5-LO product synthesis in HEK cells is also reflected from experiments with MK886, a potent FLAP antagonist with IC_{50} values in the low nanomalar range [9]. MK886 up to 1 μM failed to reduce 5-LO activity in cells expressing only 5-LO, but under conditions where FLAP seemingly stimulated 5-LO product formation (i.e., HEK-5-LO/FLAP cells activated with A23187 plus 3 μM AA) MK886 reduced it by 35% at low nanomolar concentrations. Thus, MK886 solely antagonized the 5-LO-stimulatory FLAP effect. Strong evidence that FLAP channels the first intermediate 5-HpETE towards LTA_4 is provided when examining the metabolite profile under MK886 treatment. MK886 but not the 5-LO inhibitor zileuton reduced the formation of LTA_4 as visualized by lower LTA_4 metabolites versus 5-H(p)ETE. FLAP is known to supply 5-LO with AA and is proposed to function as a membrane anchor for 5-LO [11,31], but here we demonstrate an additional activity, that is, promoting 5-LO synthase activity leading to LTA_4 at the expense of 5-HETE. It is conceivable that FLAP retains AA inside the substrate cavity of 5-LO to support hydrogen abstraction at C-7 with dioxygenation at C-5 and subsequent hydrogen abstraction at C-10 and thus LTA_4 formation.

Results from the IF microscopy analysis confirm the current understanding of 5-LO subcellular redistribution and co-localization with FLAP at the nuclear membrane [27,30,33]. Our data show that 5-LO translocated from the nucleoplasm and cytosol to the nuclear membrane in HEK-5-LO/FLAP cells after challenge with A23187 and co-localized with FLAP at the perinuclear region. As expected, 5-LO co-localization with FLAP was prevented by MK886 in cells expressing both proteins which is in agreement with MK886 actions in human leukocytes after A23187 stimulation [10,41].

In conclusion, we present a cell model that may help to study the role of FLAP as 5-LO interacting protein in LT biosynthesis in intact cells and the characterization of putative FLAP antagonists. Our data clearly show that co-expression of 5-LO and FLAP and their co-localization at the nuclear membrane together with concomitant release of AA are not sufficient for cellular LT formation from endogenous substrate, even though supplementation of low amounts of exogenous AA leads to tremendous 5-LO product synthesis. It will be challenging in future studies to reveal the respective missing piece of the puzzle. Furthermore, putative FLAP inhibitors can be better examined in such model as interference with FLAP within LT biosynthesis can be distinguished from interference with 5-LO. In fact, novel FLAP antagonists are currently evaluated in clinical trials as potential therapeutics for LT-related disease [42] and our presented cellular model may facilitate hit identification in early preclinical studies.

Acknowledgments

We thank Dr. Marcia E. Newcomer for helpful discussion during writing this manuscript.

References

[1] M. Peters-Golden, W.R. Henderson Jr., Leukotrienes, N. Engl. J. Med. 357 (2007) 1841–1854.
[2] R.L. Maas, C.D. Ingram, D.F. Taber, J.A. Oates, A.R. Brash, Stereospecific removal of the DR hydrogen atom at the 10-carbon of arachidonic acid in the biosynthesis of leukotriene A4 by human leukocytes, J. Biol. Chem. 257 (1982) 13515–13519.
[3] A. Panossian, M. Hamberg, B. Samuelsson, On the mechanism of biosynthesis of leukotrienes and related compounds, FEBS Lett. 150 (1982) 511–513.
[4] C.N. Serhan, M. Hamberg, B. Samuelsson, Lipoxins: novel series of biologically active compounds formed from arachidonic acid in human leukocytes, Proc. Natl. Acad. Sci. U. S. A. 81 (1984) 5335–5339.
[5] M. Minami, S. Ohno, H. Kawasaki, O. Radmark, B. Samuelsson, H. Jornvall, T. Shimizu, Y. Seyama, K. Suzuki, Molecular cloning of a cDNA coding for human leukotriene A4 hydrolase. Complete primary structure of an enzyme involved in eicosanoid synthesis, J. Biol. Chem. 262 (1987) 13873–13876.
[6] J.F. Penrose, LTC4 synthase. Enzymology, biochemistry, and molecular characterization, Clin. Rev. Allergy Immunol. 17 (1999) 133–152.
[7] M. Rakonjac, L. Fischer, P. Provost, O. Werz, D. Steinhilber, B. Samuelsson, O. Radmark, Coactosin-like protein supports 5-lipoxygenase enzyme activity and up-regulates leukotriene A4 production, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 13150–13155.
[8] O. Werz, E. Burkert, L. Fischer, D. Szellas, D. Dishart, B. Samuelsson, O. Radmark, D. Steinhilber, 5-Lipoxygenase activation by MAPKAPK-2 and ERKs, Adv. Exp. Med. Biol. 525 (2003) 129–132.
[9] R.A. Dixon, R.E. Diehl, E. Opas, E. Rands, P.J. Vickers, J.F. Evans, J.W. Gillard, D.K. Miller, Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis, Nature 343 (1990) 282–284.
[10] C.A. Rouzer, A.W. Ford-Hutchinson, H.E. Morton, J.W. Gillard, MK886, a potent and specific leukotriene biosynthesis inhibitor blocks and reverses the membrane association of 5-lipoxygenase in ionophore-challenged leukocytes, J. Biol. Chem. 265 (1990) 1436–1442.
[11] J.W. Woods, J.F. Evans, D. Ethier, S. Scott, P.J. Vickers, L. Hearn, J.A. Heibein, S. Charleson, I.I. Singer, 5-Lipoxygenase and 5-lipoxygenase-activating protein are localized in the nuclear envelope of activated human leukocytes, J. Exp. Med. 178 (1993) 1935–1946.
[12] P.J. Jakobsson, R. Morgenstern, J. Mancini, A. Ford-Hutchinson, B. Persson, Membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG). A widespread protein superfamily, Am. J. Respir. Crit. Care Med. 161 (2000) S20–S24.
[13] S. Charleson, P. Prasit, S. Leger, J.W. Gillard, P.J. Vickers, J.A. Mancini, P. Charleson, J. Guay, A.W. Ford-Hutchinson, J.F. Evans, Characterization of a 5-lipoxygenase-activating protein binding assay: correlation of affinity for 5-lipoxygenase-activating protein with leukotriene synthesis inhibition, Mol. Pharmacol. 41 (1992) 873–879.
[14] S. Kargman, P.J. Vickers, J.F. Evans, A23187-induced translocation of 5-lipoxygenase in osteosarcoma cells, J. Cell Biol. 119 (1992) 1701–1709.
[15] X.S. Chen, Y.Y. Zhang, C.D. Funk, Determinants of 5-lipoxygenase nuclear localization using green fluorescent protein/5-lipoxygenase fusion proteins, J. Biol. Chem. 273 (1998) 31237–31244.
[16] O. Werz, E. Burkert, B. Samuelsson, O. Radmark, D. Steinhilber, Activation of 5-lipoxygenase by cell stress is calcium independent in human polymorphonuclear leukocytes, Blood 99 (2002) 1044–1052.
[17] C. Pergola, A. Rogge, G. Dodt, H. Northoff, C. Weinigel, D. Barz, O. Radmark, L. Sautebin, O. Werz, Testosterone suppresses phospholipase D, causing sex differences in leukotriene biosynthesis in human monocytes, FASEB J. 25 (2011) 3377–3387.
[18] O. Werz, D. Szellas, D. Steinhilber, O. Radmark, Arachidonic acid promotes phosphorylation of 5-lipoxygenase at Ser-271 by MAPK-activated protein kinase 2 (MK2), J. Biol. Chem. 277 (2002) 14793–14800.
[19] C. Pergola, G. Dodt, A. Rossi, E. Neunhoeffer, B. Lawrenz, H. Northoff, B. Samuelsson, O. Radmark, L. Sautebin, O. Werz, ERK-mediated regulation of leukotriene biosynthesis by androgens: a molecular basis for gender differences in inflammation and asthma, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 19881–19886.
[20] P. Borgeat, B. Samuelsson, Arachidonic acid metabolism in polymorphonuclear leukocytes: effects of ionophore A23187, Proc. Natl. Acad. Sci. U. S. A. 76 (1979) 2148–2152.
[21] D. Poeckel, L. Tausch, N. Kather, J. Jauch, O. Werz, Boswellic acids stimulate arachidonic acid release and 12-lipoxygenase activity in human platelets independent of $Ca2^+$ and differentially interact with platelet-type 12-lipoxygenase, Mol. Pharmacol. 70 (2006) 1071–1078.
[22] J. Gillard, A.W. Ford-Hutchinson, C. Chan, S. Charleson, D. Denis, A. Foster, R. Fortin, S. Leger, C.S. McFarlane, H. Morton, et al., L-663,536 (MK-886) (3-[1-(4-chlorobenzyl)-3-t-butyl-thio-5-isopropylindol-2-yl]-2,2 - dimethylpropanoic acid), a novel, orally active leukotriene biosynthesis inhibitor, Can. J. Physiol. Pharmacol. 67 (1989) 456–464.
[23] S. Charleson, J.F. Evans, S. Leger, H. Perrier, P. Prasit, Z. Wang, P.J. Vickers, Structural requirements for the binding of fatty acids to 5-lipoxygenase-activating protein, Eur. J. Pharmacol. 267 (1994) 275–280.
[24] G.W. Carter, P.R. Young, D.H. Albert, J. Bouska, R. Dyer, R.L. Bell, J.B. Summers, D.W. Brooks, 5-lipoxygenase inhibitory activity of zileuton, J. Pharmacol. Exp. Ther. 256 (1991) 929–937.
[25] O. Radmark, O. Werz, D. Steinhilber, B. Samuelsson, 5-Lipoxygenase: regulation of expression and enzyme activity, Trends Biochem. Sci. 32 (2007) 332–341.
[26] M.E. Surette, R. Palmantier, J. Gosselin, P. Borgeat, Lipopolysaccharides prime whole human blood and isolated neutrophils for the increased synthesis of 5-lipoxygenase products by enhancing arachidonic acid availability: involvement of the CD14 antigen, J. Exp. Med. 178 (1993) 1347–1355.

[27] J.F. Evans, A.D. Ferguson, R.T. Mosley, J.H. Hutchinson, What's all the FLAP about?: 5-lipoxygenase-activating protein inhibitors for inflammatory diseases, Trends Pharmacol. Sci. 29 (2008) 72–78.
[28] N.S. Stock, G. Bain, J. Zunic, Y. Li, J. Ziff, J. Roppe, A. Santini, J. Darlington, P. Prodanovich, C.D. King, C. Baccei, C. Lee, H. Rong, C. Chapman, A. Broadhead, D. Lorrain, L. Correa, J.H. Hutchinson, J.F. Evans, P. Prasit, 5-Lipoxygenase-activating protein (FLAP) inhibitors. Part 4: development of 3-[3-tert-butylsulfanyl-1-[4-(6-ethoxypyridin-3-yl)benzyl]-5-(5-methylpyridin-2-y lmethoxy)-1H-indol-2-yl]-2,2-dimethylpropionic acid (AM803), a potent, oral, once daily FLAP inhibitor, J. Med. Chem. 54 (2011) 8013–8029.
[29] A.K. Mandal, J. Skoch, B.J. Bacskai, B.T. Hyman, P. Christmas, D. Miller, T.T. Yamin, S. Xu, D. Wisniewski, J.F. Evans, R.J. Soberman, The membrane organization of leukotriene synthesis, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 6587–6592.
[30] A.K. Mandal, P.B. Jones, A.M. Bair, P. Christmas, D. Miller, T.T. Yamin, D. Wisniewski, J. Menke, J.F. Evans, B.T. Hyman, B. Bacskai, M. Chen, D.M. Lee, B. Nikolic, R.J. Soberman, The nuclear membrane organization of leukotriene synthesis, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 20434–20439.
[31] M. Abramovitz, E. Wong, M.E. Cox, C.D. Richardson, C. Li, P.J. Vickers, 5-Lipoxygenase-activating protein stimulates the utilization of arachidonic acid by 5-lipoxygenase, Eur. J. Biochem. 215 (1993) 105–111.
[32] J.A. Mancini, M. Abramovitz, M.E. Cox, E. Wong, S. Charleson, H. Perrier, Z. Wang, P. Prasit, P.J. Vickers, 5-Lipoxygenase-activating protein is an arachidonate binding protein, FEBS Lett. 318 (1993) 277–281.
[33] A.M. Bair, M.V. Turman, C.A. Vaine, R.A. Panettieri Jr., R.J. Soberman, The nuclear membrane leukotriene synthetic complex is a signal integrator and transducer, Mol. Biol. Cell 23 (2012) 4456–4464.
[34] N.C. Gilbert, S.G. Bartlett, M.T. Waight, D.B. Neau, W.E. Boeglin, A.R. Brash, M.E. Newcomer, The structure of human 5-lipoxygenase, Science 331 (2011) 217–219.
[35] A.D. Ferguson, B.M. McKeever, S. Xu, D. Wisniewski, D.K. Miller, T.T. Yamin, R.H. Spencer, L. Chu, F. Ujjainwalla, B.R. Cunningham, J.F. Evans, J.W. Becker, Crystal structure of inhibitor-bound human 5-lipoxygenase-activating protein, Science 317 (2007) 510–512.
[36] O. Kifor, R. Diaz, R. Butters, E.M. Brown, The Ca^{2+}—sensing receptor (CaR) activates phospholipases C, A2, and D in bovine parathyroid and CaR-transfected, human embryonic kidney (HEK293) cells, J. Bone Miner. Res. Off. J. Am. Soc. Bone Miner. Res. 12 (1997) 715–725.
[37] C. Pergola, J. Gerstmeier, B. Monch, B. Caliskan, S. Luderer, C. Weinigel, D. Barz, J. Maczewsky, S. Pace, A. Rossi, L. Sautebin, E. Banoglu, O. Werz, The novel benzimidazole derivative BRP-7 inhibits leukotriene biosynthesis in vitro and in vivo by targeting 5-lipoxygenase-activating protein (FLAP), Br. J. Pharmacol. 171 (12) (2014) 3051–3064.
[38] D. Steinhilber, S. Hoshiko, J. Grunewald, O. Radmark, B. Samuelsson, Serum factors regulate 5-lipoxygenase activity in maturating HL60 cells, Biochim. Biophys. Acta 1178 (1993) 1–8.
[39] L. Fischer, M. Hornig, C. Pergola, N. Meindl, L. Franke, Y. Tanrikulu, G. Dodt, G. Schneider, D. Steinhilber, O. Werz, The molecular mechanism of the inhibition by licofelone of the biosynthesis of 5-lipoxygenase products, Br. J. Pharmacol. 152 (2007) 471–480.
[40] C.A. Rouzer, E. Rands, S. Kargman, R.E. Jones, R.B. Register, R.A. Dixon, Characterization of cloned human leukocyte 5-lipoxygenase expressed in mammalian cells, J. Biol. Chem. 263 (1988) 10135–10140.
[41] M.E. Newcomer, N.C. Gilbert, Location, location, location: compartmentalization of early events in leukotriene biosynthesis, J. Biol. Chem. 285 (2010) 25109–25114.
[42] D. Singh, M. Boyce, V. Norris, S.E. Kent, J.H. Bentley, Inhibition of the early asthmatic response to inhaled allergen by the 5-lipoxygenase activating protein inhibitor GSK2190915: a dose–response study, Int. J. Gen. Med. 6 (2013) 897–903.

3.3 Manuscript III

Time-resolved *in situ* assembly of the leukotriene-synthetic 5-LO / FLAP complex in blood leukocytes

Gerstmeier, J., C. Weinigel, S. Rummler, O. Rådmark, O. Werz and U. Garscha (2015)

FASEB J, under review after invitation to submit.

The assembly of 5-LO, FLAP, and CLP in activated human leukocytes at the nuclear membrane is pivotal for cellular LT biosynthesis. For the first time, we present the time-resolved subcellular localization of these proteins, concomitant with LT biosynthesis, and provide distinct proof for the *in situ* protein-protein interaction of native 5-LO and FLAP in stimulated leukocytes, by proximity ligation assay. 5-LO/FLAP interaction occurs delayed compared to LT formation and 5-LO translocation, in contrast to the continuous, stimulus-independent 5-LO/CLP interaction. FLAP antagonists and inhibition of AA release abolished the 5-LO/FLAP assembly that was restored by exogenous AA and 5-HPETE without effecting 5-LO translocation and co-localization with FLAP. Though neutrophils and monocytes possess comparable quantities of catalytic active 5-LO enzyme, 5-LO activity in intact neutrophils is pronounced due to prolonged cellular 5-LO reaction, accompanied by delayed 5-LO nuclear membrane translocation. There is evidence that FLAP rather “regulates” cellular LT biosynthesis than simply “activates” it.

Contribution (80%): Purification of human monocytes and neutrophils, 5-LO activity assay in intact cells and cell homogenates, HPLC analysis of 5-LO products, stable transfection of HEK293 cells, immunofluorescence microscopy, proximity ligation assay, [^{3}H]-AA release, SDS-page and Western blot, data analysis and preparation of graphs, analysis of statistics, writing the manuscript.

Time-resolved *in situ* assembly of the leukotriene-synthetic 5-LO / FLAP complex in blood leukocytes

Short title: ***In situ* assembly of the 5-LO / FLAP complex**

Jana Gerstmeier[1], Christina Weinigel[2], Silke Rummler[2], Olof Rådmark[3], Oliver Werz[1], Ulrike Garscha[1]

[1]Chair of Pharmaceutical/Medicinal Chemistry, Institute of Pharmacy, Friedrich-Schiller-University, [2]Institute of Transfusion Medicine, Jena University Hospital, 07743 Jena, Germany, [3]Department of Medical Biochemistry and Biophysics, Division of Physiological Chemistry II, Karolinska Institutet, Stockholm, Sweden

Corresponding author:

Ulrike Garscha; Chair of Pharmaceutical/Medicinal Chemistry, Institute of Pharmacy, Friedrich-Schiller-University; Philosophenweg 14; 07743 Jena; Germany Tel.: +49 3641 949811; Fax: +4903641 949802 mail: ulrike.garscha@uni-jena.de

Oliver Werz; Chair of Pharmaceutical/Medicinal Chemistry, Institute of Pharmacy, Friedrich-Schiller-University; Philosophenweg 14; 07743 Jena; Germany Tel.: +49 3641 949801; Fax: +4903641 949802 mail: oliver.werz@uni-jena.de

Abstract

5-Lipoxygenase (5-LO) catalyzes the initial steps in the biosynthesis of pro-inflammatory leukotrienes (LTs). Upon cell activation 5-LO translocates to the nuclear membrane where arachidonic acid is transferred by 5-LO-activating protein (FLAP) to 5-LO for metabolism. Although previous data indicate association of 5-LO with FLAP, the *in situ* assembly of native 5-LO/FLAP complexes remains elusive. Here, we show time-resolved 5-LO/FLAP co-localization by immunofluorescence microscopy and *in situ* 5-LO/FLAP interaction by proximity ligation assay at the nuclear membrane of Ca^{2+}-ionophore A23187-activated human monocytes and neutrophils in relation to 5-LO activity. While 5-LO translocation and product formation is completed within 1.5-3 min, 5-LO/FLAP interaction is delayed and proceeds up to 30 min. Though monocytes and neutrophils contain comparable amounts of 5-LO protein, neutrophils produce 3-5 times higher levels of 5-LO products due to prolonged activity, accompanied by delayed 5-LO nuclear membrane translocation. Arachidonic acid seemingly acts as adaptor for 5-LO/FLAP assembly, whereas FLAP inhibitors (MK886, 100 nM; BAY X 1005, 3 µM) disrupt the complex. We conclude that FLAP may regulate 5-LO activity in two ways: first by inducing an initial flexible association for efficient 5-LO product synthesis, followed by the formation of a tight 5-LO/FLAP complex that terminates 5-LO activity.

Abbreviations:

5-LO, 5-lipoxygenase; 5-H(p)ETE, 5(S)-hydro(pero)xy-6-trans-8,11,14-cis-eicosatetraenoic acid; AA, arachidonic acid; CLP, coactosin-like protein; FLAP, 5-LO-activating protein; IF, immunofluorescence; LT, leukotriene; NM, nuclear membrane; PC, phosphatidylcholine; PGC buffer, PBS, 0.1% glucose, 1 mM $CaCl_2$; PLA, proximity ligation assay;

Key words:

eicosanoids, proximity-ligation assay, lipoxygenases, inflammation

Introduction

The human immune system is a highly developed and complex orchestra of pro-inflammatory and anti-inflammatory mediators to assure host defense, but excessive and non-resolving inflammation form the basis of the pathophysiology of diseases like asthma, cancer and atherosclerosis (1-3). Leukotrienes (LTs) are pivotal pro-inflammatory lipid mediators that are synthesized from arachidonic acid (AA) involving 5-lipoxygenase (5-LO) (4). First, 5-LO catalyzes the incorporation of molecular oxygen at C5 of AA yielding the intermediate 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HpETE) that in the second step is dehydrated by 5-LO to LTA_4 (5). The cellular capacity to generate 5-H(p)ETE and LTA_4 depends on the translocation of 5-LO from a soluble compartment to the nuclear membrane (NM), regulated by a fine-tuned machinery involving Ca^{2+}-mediated binding to phosphatidylcholine (PC) in the NM, phosphorylation at serine residues (6-10), and interaction of 5-LO with coactosin-like protein (CLP) (11, 12).

In addition, cellular LT formation from endogenous AA is determined by the NM-bound 5-LO-activating protein (FLAP) that may interact with 5-LO (13). It is assumed that FLAP acts as scaffold for 5-LO that governs the distribution of 5-LO to the perinuclear region, thus enabling efficient LTA_4 formation at this locale (14, 15). The requirement of FLAP in intact cells is unique and indispensable for 5-LO product synthesis (but dispensable for other LOs), and FLAP appears to function as an AA transfer protein for 5-LO, devoid of enzymatic activity (16, 17). Genetic knockdown or inhibition of FLAP prevents LT formation in cell-based assays and *in vivo*, and FLAP inhibitors were reported to interfere with nuclear translocation of 5-LO (12, 18). However, the proposed interaction between 5-LO and FLAP in intact human leukocytes, concomitant to 5-LO product synthesis, is still obscure and if additional factors regulate this interaction is elusive. Despite

in vitro pull-down assays with artificial GST-tagged FLAP and 5-LO (19), co-immunoprecipitation experiments (20) or association studies using membrane-permanent cross-linking agents to perform fluorescence lifetime imaging microscopy (FLIM) in mouse neutrophils and rat RBL-2H3 cell lines (20), there has been no direct proof for an interaction of the two native proteins in intact human leukocytes, until now. Furthermore, we emphasize that subcellular co-localization of two proteins, visualized by immunofluorescence microscopy (IF), does not necessarily imply that a tight and/or functional protein-protein interaction occurs. Here, we investigated the subcellular localization of 5-LO and the *in situ* interaction between native 5-LO and FLAP as well as 5-LO product synthesis in stimulated human blood leukocytes. Our study is the first that addresses the time-resolved *in situ* assembly of native 5-LO/FLAP complexes in primary human monocytes and neutrophils, and suggests a regulatory rather than a purely activating function of FLAP in 5-LO product synthesis.

Materials and Methods

Materials

MK886 and 5(*S*)-HPETE were from Cayman Chemicals (Ann Arbor, US); BAY-X 1005, Tocris Bioscience (Bristol, UK); zileuton, Sequoia Research Products (Oxford, UK); RSC-3388 and mowiol were from Calbiochem (Bad Soden, Germany). Rotiszint® eco plus, Roth (Karlsruhe, Germany). Non-immune goat serum (10%), hygromycine, geneticine, Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 555 goat anti-mouse and diamidino-2-phenylindol (DAPI) were from Invitrogen (Darmstadt, Germany). Mouse anti-5-LO monoclonal antibody was a generous gift by Dr. Dieter Steinhilber (Goethe University Frankfurt, Germany). Rabbit anti-CLP polyclonal antibody, Acris Antibodies (San Diego, CA). Rabbit anti-FLAP polyclonal antibody, Abcam (Cambridge, UK). Mouse anti-actin monoclonal antibody, Santa Cruz (Heidelberg, Germany). Tritium labeled [5,6,8,9,11,12,14,15-^{3}H]AA, Biotrend Chemicals GmbH (Cologne, Germany). HPLC solvents, Merck (Darmstadt, Germany). Poly-D-lysin coated glass coverslips, neuVitro (El Monte, CA). Duolink detection reagents red, Duolink PLA probe anti-rabbit PLUS, Duolink PLA probe anti-mouse MINUS as well as Duolink wash buffers, and all other chemicals were from Sigma-Aldrich (Deisenhofen, Germany) unless stated otherwise.

Cells

Human neutrophils and monocytes form healthy adult female donors were freshly isolated from leukocyte concentrates, as described (21). HEK293 cells were cultured as monolayers and cells expressing FLAP and/or 5-LO were selected by 200 µg/ml hygromycin B and/or 400 µg/ml geneticin, respectively, as described (15).

Determination of 5-LO product formation in neutrophils and monocytes

Cells were resuspended in PGC buffer (PBS, 0.1% glucose, 1 mM $CaCl_2$), pre-warmed and stimulated with 2.5 µM A23187 with or without AA at 37 °C for indicated periods. Acidified PBS and the internal standard (200 ng PGB_1) were added and subjected to solid phase extraction on C18-columns (100 mg, UCT, Bristol, PA). 5-LO products were isolated and quantified by RP-HPLC as described (22). 5-LO products include LTB_4, its all-trans isomers and 5-H(p)ETE. To determine 5-LO products in homogenates, cells were resuspended in ice cold PBS containing 1 mM EDTA, and sonicated for 3 × 10 sec, at 4 °C. Samples (corresponding to 1 × 10^6 cells/ml) were treated with 2 mM $CaCl_2$ and 3 µM AA at 37 °C for indicated periods. 5-LO products were analyzed as described above.

Analysis of 5-LO redistribution by immunofluorescence microscopy

HEK293 cells expressing FLAP and/or 5-LO were analyzed as described before (15). Neutrophils were pre-incubated with test compounds or vehicle for 10 min at 37 °C in PGC buffer and centrifuged onto poly-D-lysine coated glass coverslips (10 × g for 2 sec). For monocyte analysis, PBMC were seeded onto glass coverslips in a 12-well plate and cultured for 1.5 h. Cells were activated for the indicated time points at 37 °C and stopped by fixation with 4% paraformaldehyde solution. Acetone (3 min, 4 °C) and 0.25% Triton X-100 (10 min) were used for permeabilization before blocking with non-immune goat serum. Samples were incubated with mouse monoclonal anti-5-LO antibody (1:100) and rabbit polyclonal anti-FLAP antibody (5 µg/ml) at 4 °C. 5-LO and FLAP were stained with the fluorophore-labeled secondary antibodies; Alexa Fluor 488 goat anti-rabbit (1:1000) and Alexa Fluor 555 goat anti-mouse (1:1000). Nuclear DNA was stained with DAPI. Samples were analyzed by a Zeiss Axiovert 200M microscope, and a Plan Neofluar ×100/1.30 Oil (DIC III) objective (Carl Zeiss, Jena, Germany). An AxioCam MR camera (Carl Zeiss) was used for image acquisition

Analysis of protein interaction by in situ proximity ligation assay

To detect *in situ* interaction of 5-LO with FLAP or CLP in neutrophils, monocytes, and HEK293 cells, an *in situ* proximity ligation assay (PLA) was performed, according the manufacturer´s protocol (23). Cells were treated, fixed and incubated with primary antibody as described for IF microscopy. The cells were incubated with species specific secondary antibodies conjugated with oligonucleotides (PLA probe anti-mouse MINUS and anti-rabbit PLUS) for 1 h at 37 °C. By addition of two other circle-forming DNA oligonucleotides and a ligase (30 min at 37 °C) the antibody-bound oligonucleotides form a DNA circle when the target proteins are less than 40 nm distant from each other. The newly generated DNA circle was amplified by rolling circle amplification, and

visualized by hybridization with fluorescently labeled oligonucleotides. Nuclear DNA was stained with DAPI. The PLA interaction signal appears as a fluorescent spot and was analyzed by fluorescence microscopy using a Zeiss Axiovert 200M microscope, and a Plan Neofluar ×100/1.30 Oil (DIC III) objective as well as a Plan Neofluar ×40/1.30 Oil (DIC III) objective (Carl Zeiss, Jena, Germany).

Determination of release of [^{3}H]-labeled arachidonic acid

Release of [^{3}H]-labeled AA from human leukocytes was analyzed as described (24). In brief, cells were resuspended in medium and incubated with 5 nM [^{3}H]AA (corresponding to 0.5 µCi/ml, specific activity 200 Ci/mmol) for 2 h at 37 °C. Cells were collected, washed to remove unincorporated [^{3}H]AA, and resuspended in PBS containing 1 mM $CaCl_2$. The cells were pre-incubated with 1 µM RSC-3388 (a cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) inhibitor) or vehicle (0.1% DMSO) at 37 °C for 15 min, and subsequently stimulated with 2.5 µM A23187 for 10 min at 37 °C. The reaction was stopped on ice (10 min) and cells were centrifuged. Aliquots of the supernatants were combined with 2 ml Rotiszint® eco plus and assayed for radioactivity by scintillation counting (Micro Beta Trilux, Perkin Elmer, Waltham, MA).

SDS-PAGE and Western blot analysis

Cell lysates of neutrophils and monocytes, corresponding to 5×10^6 cells, were separated on 10% (5-LO) and 16% (FLAP) polyacrylamide gels, and blotted onto nitrocellulose membranes (Hybond ECL, GE Healthcare, Freiburg, Germany). The membranes were incubated with primary antibodies (mouse anti-5-LO, 1:1000; rabbit anti-FLAP, 0.1 µg/ml and mouse anti-actin, 1:1000) with subsequent detection using IRDye 800CW-labeled anti-rabbit and/or anti-mouse antibodies (1:10,000 each). Immunoreactive bands were visualized by an Odyssey infrared imager (LI-COR Biosciences).

Statistics

Results are expressed as means ± standard error of the mean (SEM) of n observations, where n represents the number of experiments performed at different days in duplicates. Pictures of the fluorescence microscopy experiments are representative for at least three independent experiments. Graphs were generated with GraphPad Prism 4 and validated with GraphPad InStat (Graphpad Software Inc., San Diego, CA). A P-value of <0.05 (*) was considered significant using two-tailed Student t test when comparing two groups.

Results

In situ interaction of 5-LO and FLAP

In order to demonstrate the proposed interaction of native 5-LO and FLAP *in situ*, we utilized PLA. The PLA is a valuable tool for monitoring direct protein-protein interaction of native proteins at distinct locales in intact cells, without the necessity of artificial protein tags or labels (23). Oligonucleotides, attached to secondary antibodies recognizing the primary antibodies, ligates to a new circular DNA only when target proteins are in close proximity (< 40 nM). The DNA circle can be amplified and visualized by hybridization with a fluorophore (magenta). First, we utilized HEK293 cells that were stably transfected with recombinant FLAP and/or 5-LO, and that are able to generate abundant 5-LO products when both 5-LO and FLAP are expressed (15). HEK293 cells were studied for co-localization of 5-LO and FLAP (Fig. 1 A) as well as for 5-LO/FLAP *in situ* interaction (Fig. 1 B). Moreover, we took advantage of the 5-LO_3W mutant (where W13, W75, and W102 are replaced by alanine) which cannot bind to PC and CLP (11, 25) and thus, fails to translocate to the nuclear membrane. 5-LO (wild type) in resting HEK293 cells was intranuclear, regardless of the presence of FLAP. Upon activation, 5-LO translocated to the NM in cells without FLAP but the presence of FLAP enriched the portion of NM-associated 5-LO. On the other hand, 5-LO_3W resided in the cytosol of stimulated cells, also in the presence of FLAP, implying that this mutant-5-LO may not co-localize with FLAP. As expected, the PLA revealed no positive signal in HEK293 cells lacking FLAP, regardless of A23187 stimulation. However, co-expression of FLAP caused a strong PLA signal but only upon A23187 stimulation. In agreement with the data mentioned above, no positive PLA signal was obtained in stimulated HEK293 cells expressing FLAP and5-LO_3W. These results validate the reliability of the PLA as tool to visualize the cellular interaction of native 5-LO and FLAP *in situ*.

Time-resolved co-localization and interaction of 5-LO and FLAP in intact cells

Next, we aimed to analyze the interaction of native 5-LO and FLAP in primary human monocytes and neutrophils that are major sources for LT biosynthesis (26). 5-LO activity, 5-LO translocation, and interaction of 5-LO with FLAP in these leukocytes are commonly analyzed 10 to 30 min upon stimulation (20, 22, 27), but the exact time course and correlation of these processes and are unknown. Therefore, we assessed the subcellular localization of 5-LO and FLAP in monocytes (Fig. 2 A) and neutrophils (Fig. 2 B) in a time-resolved manner. FLAP, was consistently detectable at the NM (Fig. 2 A, B) as expected (28). In monocytes, intranuclear 5-LO (resting cells, t = 0) started to co-localize with FLAP already 30 sec upon A23187 exposure, and was essentially NM-associated after 60 sec up to 30 min. Interestingly, for neutrophils, 5-LO required about 90 sec to co-localize with FLAP upon A23187 stimulation, and maximal co-localization was evident after 180 sec,

without further enrichment up to 30 min. Using PLA, no 5-LO/FLAP interaction signal was obtained in unstimulated monocytes and neutrophils (Fig 3). However, 3 min upon A23187-stimulation, a 5-LO/FLAP complex was formed at the perinuclear region of monocytes and neutrophils (Fig. 3). It seems that 5-LO/FLAP complex formation appeared delayed compared to 5-LO/FLAP co-localization and increased up to 30 min. In contrast, an interaction between 5-LO and CLP, analyzed as control, was observed in both resting and activated cells (see Supplementary Fig. 1).

FLAP inhibitors do not interfere with 5-LO translocation but disrupt the 5-LO/FLAP interaction

To determine the influence of LT biosynthesis inhibitors on 5-LO/FLAP co-localization, cells were pretreated with the FLAP inhibitors MK886 (300 nM) and BAY X-1005 (3 μM) as well as with the 5-LO inhibitor zileuton (10 μM) and the cPLA$_2$α inhibitor RSC-3388 (5 μM) (Fig. 2 C). Cells were then stimulated for 3 min with A23187 and subcellular localization of 5-LO and FLAP was analyzed. Zileuton and RSC-3388 failed to influence the localization pattern of 5-LO in resting or A23187-activated cells. Of interest, MK886 and BAY X-1005 only marginally diminished 5-LO co-localization with FLAP in monocytes and neutrophils (Fig. 2 C). Nevertheless, we anticipated that FLAP inhibitors may instead efficiently interrupt the 5-LO/FLAP interaction. Therefore, the effects of the FLAP inhibitors were assessed by PLA in monocytes and neutrophils. MK886 and BAY X-1005 entirely abolished the PLA signal of A23187-stimulated monocytes and neutrophils (Fig. 4), suggesting that the 5-LO/FLAP interaction is disrupted. The direct 5-LO inhibitor zileuton did not impair the 5-LO/FLAP interaction (Fig. 4), as expected.

AA or its metabolite 5-HPETE is required for the 5-LO/FLAP interaction

Previous data propose a central role of AA in the assembly of the 5-LO/FLAP complex (27) and both 5-LO and FLAP can bind AA (16). Pretreatment of monocytes or neutrophils with the cPLA$_2$α inhibitor RSC-3388 (5 μM) prior to A23187 stimulation completely abolished the signal in the PLA suggesting that lack of endogenously-released AA prevents 5-LO/FLAP complex formation (Fig. 5). In fact, rescue experiments using exogenous AA (10 μM) or the 5-LO intermediate 5-HpETE (5 μM) reversed the inhibitory effects of RSC-3388 and enabled 5-LO/FLAP interaction (Fig. 5). These findings strongly suggest a modulating role for AA and/or 5-HpETE in the assembly of the LT biosynthetic 5-LO/FLAP complex at the nuclear membrane.

Cellular 5-LO product formation in human leukocytes

In order to correlate co-localization and interaction of 5-LO with FLAP to 5-LO activity, we analyzed

the ability of human neutrophils and monocytes to form 5-LO products upon A23187 stimulation (Fig. 6 A). Within 10 min, neutrophils formed about 4- to 5-times higher amounts of 5-LO products than monocytes, which might be simply due to higher AA substrate supply in neutrophils. In fact, when stimulated with A23187, more robust release of AA and its metabolites was evident for [^{3}H]AA-labelled neutrophils as compared to [^{3}H]AA-labelled monocytes (Fig. 6 B). The cPLA$_2$ inhibitor RSC-3388 suppressed release of [^{3}H]AA and its metabolites in both cell types. To circumvent cPLA$_2$-mediated AA supply as limiting factor for 5-LO product synthesis, we stimulated both cell types with exogenous AA (3, 10 or 30 µM) and A23187. Again, 5-LO product synthesis was 3- to 5-fold higher in neutrophils versus monocytes, with a peak at 10 µM AA in both cell types. The amounts of 5-LO protein in neutrophils and monocytes were similar, analyzed side-by-side by Western blotting (normalized to actin), but FLAP was significantly more abundant in neutrophils (Fig. 6 C/D). In line with these findings, 5-LO product synthesis in homogenates in the presence of 3 µM AA (which may resemble the AA concentration in intact A23187-stimulated cells (29)), were comparable between the two cell types. We next analyzed the time-resolved 5-LO product synthesis from endogenous AA in A23187-activated neutrophils and monocytes. 5-LO product synthesis in monocytes was essentially complete after about 60-90 sec of stimulation without significant differences in the amounts of 5-LO products formed in neutrophils at this time point (Fig. 6 E). In neutrophils however, 5-LO product synthesis continued up to 180 sec resulting in 4-fold higher amounts of formed metabolites versus monocytes (Fig. 6 E). When cells were stimulated with A23187 in the presence of 10 µM exogenous AA, a rapid onset and progression of 5-LO product synthesis was seen, being essentially complete after 60-90 sec in both cell types (Fig. 6 F). Note that in homogenates of neutrophils and monocytes (Fig. 6 G) the time-resolved 5-LO reaction was similar, proceeding up to 60-90 sec with comparable quantities of 5-LO products formed. Conclusively, A23187-induced 5-LO product formation in monocytes and neutrophils is rapid, and the higher capacity of 5-LO products in neutrophils seems to be due to a prolonged 5-LO reaction along with delayed 5-LO/FLAP co-localization as compared to monocytes.

Discussion

The biosynthesis of LTs is tightly compartmentalized, with enzymes distributed in the cytosol, nucleoplasm, extracellular space as well as at the nuclear membrane (30). The undisputed role of FLAP for efficient cellular LT formation is known for more than two decades (13), but evidence for the proposed interaction between native 5-LO and FLAP in human leukocytes is lacking. Here, we investigated the subcellular distribution and *in situ* interaction of 5-LO and FLAP in relation to 5-LO product formation in a time-resolved manner in A23187-activated human neutrophils and monocytes.

Among blood leukocytes, monocytes and neutrophils possess the highest capacities to form 5-LO products (31-33). The side-by-side comparison of monocytes and neutrophils for 5-LO product synthesis in this study revealed similar amounts of 5-LO protein in both cell types. Accordingly, 5-LO product synthesis in crude homogenates of neutrophils and monocytes, where 5-LO activity is largely independent of cell type-specific regulatory mechanisms (34), followed similar kinetics and comparable amounts of 5-LO products were formed. However, 3-5-fold higher quantities of 5-LO products were formed in intact neutrophils versus monocytes after A23187 activation. Note that neutrophils expressed higher levels of FLAP than monocytes, and FLAP stimulated 5-LO product synthesis only in intact cells (14). The higher quantities of liberated AA (including it metabolites) in neutrophils implies better substrate supply and may account for enhanced 5-LO product synthesis in neutrophils. However, when exogenous AA was supplied 5-LO product formation was still 2-5-times higher in neutrophils versus monocytes. Kinetic analysis suggests significantly prolonged 5-LO reaction from endogenous AA in neutrophils (up to 180 sec) while in monocytes a rapid onset and inactivation of 5-LO (60-90 sec) occurred. It appears that the prolonged 5-LO reaction in neutrophils accounts for higher capacities of 5-LO products synthesized. As A23187 causes immediate and receptor-independent elevation of intracellular Ca^{2+} levels that activate 5-LO (35) and thereby circumvents complex signaling, we used this stimulus to rule out any cell-type specific fine tuning for unbiased induction of 5-LO product formation in monocytes and neutrophils.

The kinetics of 5-LO translocation to the NM strongly correlated with 5-LO product synthesis, and it appears that the bulk of 5-LO products formed occurs during the period while 5-LO moves. Thus, in monocytes 5-LO NM-association was more rapid, occurred within 60-90 sec, and 5-LO product synthesis was complete after 90 sec. In neutrophils, 5-LO NM-association was delayed and continuously increased up to 180 sec, which in fact was in line with the prolonged 5-LO product formation in neutrophils for 180 sec. Termination of cellular 5-LO product biosynthesis is seemingly due to irreversible turnover-based suicide inactivation of 5-LO (36, 37). The faster translocation of 5-LO to the NM in monocytes may lead to accelerated inactivation of 5-LO and thus lower formation of total amounts of 5-LO products. For purified 5-LO in the presence of PC vesicles, the rate of inactivation primarily depends on substrate accessibility and substrate concentration, but also the presence of lipid peroxides in phospholipids which 5-LO binds during activation can probably compromise 5-LO by non-turnover inactivation (38). Conclusively, our data suggest that the higher cellular 5-LO product synthesis in neutrophils is due to prolonged 5-LO reaction. So far, the mechanism by which 5-LO is inactivated in membranes after translocation is elusive.

IF microscopy of 5-LO and FLAP in parallel enabled co-localization studies at the NM (12, 15,

27), but whether the two proteins closely interact with each other cannot be answered using this technique. The PLA is a sophisticated method to reveal the proximity of two unmodified, native proteins (no tags, dyes, or labels) *in situ* of less than 40 nm distance, at a defined locale in the cell (23). We applied this methodology to study the 5-LO/FLAP interaction, and our data unequivocally show that upon A23187 stimulation 5-LO and FLAP interact at the NM of monocytes and neutrophils as well as of HEK cells. Of interest, the 5-LO/FLAP interaction appears earliest at 60-90 sec, time points where 5-LO product formation in monocytes was almost terminated, and the PLA signal increased over time up to 30 min in monocytes as well as in neutrophils. Possibly, 5-LO that proceeds towards the NM may first loosely associate with FLAP, not yet close enough to be detectable by PLA, where AA and 5-HpETE provision to 5-LO may occur within a highly flexible 5-LO/FLAP assembly, before a tight 5-LO/FLAP interaction is fixed and product formation is terminated. It is conceivable that the 5-LO/FLAP interaction prevails to keep 5-LO in a resting mode at the NM after LT biosynthesis took place.

The true existence of the 5-LO/FLAP complex in monocytes and neutrophils visualized by PLA was validated and supported by several controls. First, association of 5-LO and CLP was also monitored as a continuous stimulation-independent PLA signal, binding between these proteins *in vitro* and in cells has been documented by several methods (11, 12). Second, a PLA signal due to a 5-LO/FLAP complex in HEK293 cells was evident exclusively in A23187-activated cells expressing both 5-LO and FLAP. Third, when HEK293 cells expressing FLAP and the 5-LO_3W mutant that failed to translocate to the NM (25), A23187 caused no PLA signal. This is may be the result of abolished binding of 5-LO_3W to CLP (11) which would be expected to disable 5-LO association with the NM (12), and to reduce PC binding (25). Finally, the two structurally distinct FLAP antagonists MK886 (18) and BAY X-1005 (39) but not the 5-LO inhibitor zileuton clearly blocked the 5-LO/FLAP interaction PLA signal.

FLAP was long suggested to facilitate substrate transfer to NM-associated 5-LO upon *in situ* release of AA by $cPLA_2$ (14, 16). Thus, FLAP promoted both the 5-LO-mediated conversion of AA to 5-HpETE and the dehydration of 5-HpETE to LTA_4 (12, 13, 15). We show that $cPLA_2$ inhibition prevents 5-LO/FLAP interaction which is in agreement with previous studies (27), and supplementation of AA or 5-HpETE restored the 5-LO/FLAP complex. Hence, AA or 5-HpETE are essential for the 5-LO/FLAP interaction possibly by acting as adaptor molecules. Alternatively, binding of AA or 5-HpETE may cause a conformational change of FLAP and/or 5-LO, which might initialize the 5-LO/FLAP interaction.

Inhibitors of FLAP are proposed to act as competitors of AA and both shared the same binding site at FLAP (40). In fact, ample supply of exogenous AA overcomes cellular 5-LO product synthesis inhibition by FLAP (24). Our data show

that FLAP antagonists efficiently prevent the 5-LO/FLAP interaction, seemingly by interference with the adaptor functionality of AA or 5-HpETE. However, these FLAP inhibitors as well as blockade of AA supply obviously failed to prevent 5-LO translocation and co-localization with FLAP at the NM. Together with the finding that 5-LO moved to the NM in HEK293 cells lacking FLAP our results imply that 5-LO binds to the NM without the aid of FLAP.

In conclusion, our results suggest that FLAP serves rather as a regulatory protein in LT formation than a purely activating protein, generating a time-dependent assembly of the LT biosynthetic complex at the NM that enables and regulates LT formation. Our data reveal significant regulatory differences in the capacity of cellular 5-LO product synthesis in monocytes and neutrophils that are related to kinetic features of 5-LO translocation to the NM. It is tempting to speculate that FLAP regulates 5-LO product synthesis in two ways: (I) by inducing an initial flexible and loose interaction with 5-LO to transfer AA to 5-LO for efficient metabolism and (II) by induction of a tight 5-LO/FLAP complex that disables the 5-LO reaction and limits 5-LO product synthesis.

Acknowledgements

We thank Heiner Atze for technical assistance. Financial support was provided by Deutsche Forschungsgemeinschaft (DFG) within the SFB1127: Chemical Mediators in complex Biosystems (ChemBioSys).

References

1. Peters-Golden, M., and Henderson, W. R., Jr. (2007) Leukotrienes. *N. Engl. J. Med.* **357**, 1841-1854
2. Wang, D., and Dubois, R. N. (2010) Eicosanoids and cancer. *Nat. Rev. Cancer* **10**, 181-193
3. Serhan, C. N. (2014) Pro-resolving lipid mediators are leads for resolution physiology. *Nature* **510**, 92-101
4. Samuelsson, B. (1983) Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science* **220**, 568-575
5. Panossian, A., Hamberg, M., and Samuelsson, B. (1982) On the mechanism of biosynthesis of leukotrienes and related compounds. *FEBS Lett.* **150**, 511-513
6. Werz, O., Burkert, E., Fischer, L., Szellas, D., Dishart, D., Samuelsson, B., Radmark, O., and Steinhilber, D. (2003) 5-Lipoxygenase activation by MAPKAPK-2 and ERKs. *Adv. Exp. Med. Biol.* **525**, 129-132
7. Hammarberg, T., Provost, P., Persson, B., and Radmark, O. (2000) The N-terminal domain of 5-lipoxygenase binds calcium and mediates calcium stimulation of enzyme activity. *J. Biol. Chem.* **275**, 38787-38793
8. Wong, A., Cook, M. N., Foley, J. J., Sarau, H. M., Marshall, P., and Hwang, S. M. (1991) Influx of extracellular calcium is required for the membrane translocation of 5-lipoxygenase and leukotriene synthesis. *Biochemistry* **30**, 9346-9354
9. Kargman, S., Vickers, P. J., and Evans, J. F. (1992) A23187-induced translocation of 5-lipoxygenase in osteosarcoma cells. *J. Cell Biol.* **119**, 1701-1709
10. Rouzer, C. A., and Samuelsson, B. (1985) On the nature of the 5-lipoxygenase reaction in human leukocytes: enzyme purification and requirement for multiple stimulatory factors. *Proc. Natl. Acad. Sci. U. S. A.* **82**, 6040-6044
11. Rakonjac, M., Fischer, L., Provost, P., Werz, O., Steinhilber, D., Samuelsson, B., and Radmark, O. (2006) Coactosin-like protein supports 5-lipoxygenase enzyme activity and up-regulates leukotriene A4 production. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 13150-13155
12. Basavarajappa, D., Wan, M., Lukic, A., Steinhilber, D., Samuelsson, B., and Radmark, O. (2014) Roles of coactosin-like protein (CLP) and 5-lipoxygenase-activating protein (FLAP) in cellular leukotriene biosynthesis. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 11371-11376
13. Dixon, R. A., Diehl, R. E., Opas, E., Rands, E., Vickers, P. J., Evans, J. F., Gillard, J. W., and Miller, D. K. (1990) Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis. *Nature* **343**, 282-284
14. Abramovitz, M., Wong, E., Cox, M. E., Richardson, C. D., Li, C., and Vickers, P. J. (1993) 5-lipoxygenase-activating protein stimulates the utilization of arachidonic acid by 5-lipoxygenase. *Eur. J. Biochem.* **215**, 105-111
15. Gerstmeier, J., Weinigel, C., Barz, D., Werz, O., and Garscha, U. (2014) An experimental cell-based model for studying the cell biology and molecular pharmacology of 5-lipoxygenase-activating protein in leukotriene biosynthesis. *Biochim. Biophys. Acta* **1840**, 2961-2969
16. Mancini, J. A., Abramovitz, M., Cox, M. E., Wong, E., Charleson, S., Perrier, H., Wang, Z., Prasit, P., and Vickers, P. J. (1993) 5-lipoxygenase-activating protein is an arachidonate binding protein. *FEBS Lett.* **318**, 277-281
17. Ferguson, A. D., McKeever, B. M., Xu, S., Wisniewski, D., Miller, D. K., Yamin, T. T., Spencer, R. H., Chu, L.,

Ujjainwalla, F., Cunningham, B. R., Evans, J. F., and Becker, J. W. (2007) Crystal structure of inhibitor-bound human 5-lipoxygenase-activating protein. *Science* **317**, 510-512

18. Rouzer, C. A., Ford-Hutchinson, A. W., Morton, H. E., and Gillard, J. W. (1990) MK886, a potent and specific leukotriene biosynthesis inhibitor blocks and reverses the membrane association of 5-lipoxygenase in ionophore-challenged leukocytes. *J. Biol. Chem.* **265**, 1436-1442
19. Strid, T., Svartz, J., Franck, N., Hallin, E., Ingelsson, B., Soderstrom, M., and Hammarstrom, S. (2009) Distinct parts of leukotriene C(4) synthase interact with 5-lipoxygenase and 5-lipoxygenase activating protein. *Biochem. Biophys. Res. Commun.* **381**, 518-522
20. Mandal, A. K., Jones, P. B., Bair, A. M., Christmas, P., Miller, D., Yamin, T. T., Wisniewski, D., Menke, J., Evans, J. F., Hyman, B. T., Bacskai, B., Chen, M., Lee, D. M., Nikolic, B., and Soberman, R. J. (2008) The nuclear membrane organization of leukotriene synthesis. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 20434-20439
21. Pergola, C., Dodt, G., Rossi, A., Neunhoeffer, E., Lawrenz, B., Northoff, H., Samuelsson, B., Radmark, O., Sautebin, L., and Werz, O. (2008) ERK-mediated regulation of leukotriene biosynthesis by androgens: a molecular basis for gender differences in inflammation and asthma. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 19881-19886
22. Werz, O., Burkert, E., Samuelsson, B., Radmark, O., and Steinhilber, D. (2002) Activation of 5-lipoxygenase by cell stress is calcium independent in human polymorphonuclear leukocytes. *Blood* **99**, 1044-1052
23. Soderberg, O., Gullberg, M., Jarvius, M., Ridderstrale, K., Leuchowius, K. J., Jarvius, J., Wester, K., Hydbring, P., Bahram, F., Larsson, L. G., and Landegren, U. (2006) Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nat Methods* **3**, 995-1000
24. Pergola, C., Gerstmeier, J., Monch, B., Caliskan, B., Luderer, S., Weinigel, C., Barz, D., Maczewsky, J., Pace, S., Rossi, A., Sautebin, L., Banoglu, E., and Werz, O. (2014) The novel benzimidazole derivative BRP-7 inhibits leukotriene biosynthesis in vitro and in vivo by targeting 5-lipoxygenase-activating protein (FLAP). *Br. J. Pharmacol.* **171**, 3051-3064
25. Kulkarni, S., Das, S., Funk, C. D., Murray, D., and Cho, W. (2002) Molecular basis of the specific subcellular localization of the C2-like domain of 5-lipoxygenase. *J. Biol. Chem.* **277**, 13167-13174
26. Werz, O. (2002) 5-lipoxygenase: cellular biology and molecular pharmacology. *Curr. Drug Targets Inflamm. Allergy* **1**, 23-44
27. Bair, A. M., Turman, M. V., Vaine, C. A., Panettieri, R. A., Jr., and Soberman, R. J. (2012) The nuclear membrane leukotriene synthetic complex is a signal integrator and transducer. *Mol. Biol. Cell* **23**, 4456-4464
28. Martinez Molina, D., Eshaghi, S., and Nordlund, P. (2008) Catalysis within the lipid bilayer-structure and mechanism of the MAPEG family of integral membrane proteins. *Curr. Opin. Struct. Biol.* **18**, 442-449
29. Brash, A. R. (2001) Arachidonic acid as a bioactive molecule. *J. Clin. Invest.* **107**, 1339-1345
30. Radmark, O., Werz, O., Steinhilber, D., and Samuelsson, B. (2014) 5-Lipoxygenase, a key enzyme for leukotriene biosynthesis in health and disease. *Biochim. Biophys. Acta*
31. Surette, M. E., Dallaire, N., Jean, N., Picard, S., and Borgeat, P. (1998) Mechanisms of the priming effect of lipopolysaccharides on the biosynthesis of leukotriene B4 in chemotactic peptide-stimulated human neutrophils. *FASEB J.* **12**, 1521-1531
32. Schaible, A. M., Koeberle, A., Northoff, H., Lawrenz, B., Weinigel, C., Barz, D., Werz, O., and Pergola, C. (2013) High capacity for leukotriene biosynthesis in peripheral blood during pregnancy. *Prostaglandins Leukot. Essent. Fatty Acids* **89**, 245-255
33. Radmark, O., Werz, O., Steinhilber, D., and Samuelsson, B. (2007) 5-Lipoxygenase: regulation of expression and enzyme activity. *Trends Biochem. Sci.* **32**, 332-341
34. Werz, O., and Steinhilber, D. (2005) Development of 5-lipoxygenase inhibitors--lessons from cellular enzyme regulation. *Biochem. Pharmacol.* **70**, 327-333
35. Rouzer, C. A., and Kargman, S. (1988) Translocation of 5-lipoxygenase to the membrane in human leukocytes challenged with ionophore A23187. *J. Biol. Chem.* **263**, 10980-10988
36. Lepley, R. A., and Fitzpatrick, F. A. (1994) Irreversible inactivation of 5-lipoxygenase by leukotriene A4. Characterization of product inactivation with purified enzyme and intact leukocytes. *J. Biol. Chem.* **269**, 2627-2631
37. Aharony, D., Redkar-Brown, D. G., Hubbs, S. J., and Stein, R. L. (1987) Kinetic studies on the inactivation of 5-lipoxygenase by 5(S)-hydroperoxyeicosatetraenoic acid. *Prostaglandins* **33**, 85-100
38. De Carolis, E., Denis, D., and Riendeau, D. (1996) Oxidative inactivation of human 5-lipoxygenase in phosphatidylcholine vesicles. *Eur. J. Biochem.* **235**, 416-423
39. Muller-Peddinghaus, R., Fruchtmann, R., Ahr, H. J., Beckermann, B., Buhner, K., Fugmann, B., Junge, B., Matzke, M., Kohlsdorfer, C., Raddatz, S., and et al. (1993) BAY X1005, a new selective inhibitor of leukotriene synthesis: pharmacology and pharmacokinetics. *J. Lipid Mediat.* **6**, 245-248
40. Vickers, P. J., Adam, M., Charleson, S., Coppolino, M. G., Evans, J. F., and Mancini, J. A. (1992) Identification of amino acid residues of 5-lipoxygenase-activating protein essential for the binding of leukotriene biosynthesis inhibitors. *Mol. Pharmacol.* **42**, 94-102

Figure legends

Fig. 1. Co-localization and interaction of 5-LO and FLAP in HEK293 cells.

(A) HEK293 cells, stably expressing recombinant 5-LO or 5-LO_3W with and without FLAP, each, were analyzed for subcellular localization by IF microscopy in resting and A23187-stimulated (2.5 µM, 37°C, 3 min) cells. 5-LO (Alexa Fluor 555, red) and FLAP (Alexa Fluor 488, green) were incubated with respective antibodies. (B) Direct protein interaction of 5-LO or 5-LO_3W and FLAP was analyzed by PLA in resting and A23187-stimulated (2.5 µM, 37°C, 10 min) cells. DAPI (blue) was used to stain the nucleus and *in situ* PLA signals (magenta dots) visualize 5-LO and FLAP interaction; scale bar = 30 µm. Results are representative for approx. 100 individual cells of three independent experiments.

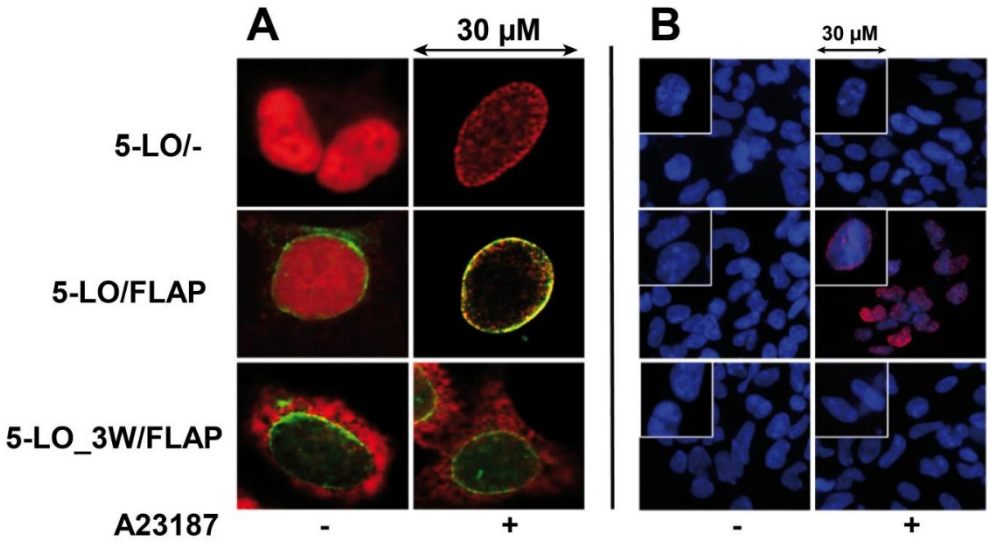


Fig. 2. Subcellular localization of 5-LO and FLAP in human leukocytes.

Localization of 5-LO upon stimulation with A23187 (2.5 µM) for the indicated time points was monitored by indirect immunofluorescence microscopy in human monocytes (A) and neutrophils (B). Cells were fixed, permeabilized and incubated with antibodies against 5-LO (Alexa Fluor 555, red) and FLAP (Alexa Fluor 488, green). (C) Preincubation for 10 min at 37 °C with zileuton (10 µM), MK886 (300 nM), BAY X-1005 (3 µM), and RSC-3388 (5 µM) before 3 min A23187 (2.5 µM) stimulation at 37 °C. Images show single staining for 5-LO (top lane), FLAP (middle lane), and overlay of 5-LO and FLAP (bottom lane). Results are representative for 100 individual cells of three independent experiments; scale bar = 10 µm (monocytes, A/C) and 15 µm (neutrophils, B/C).

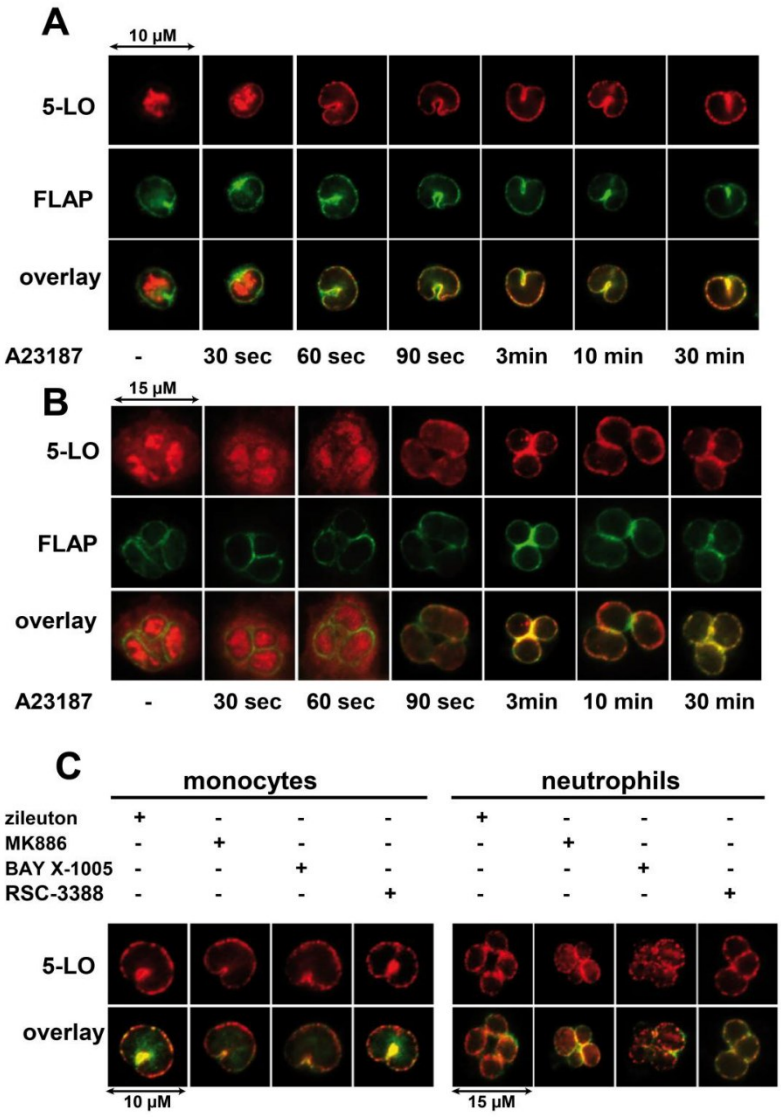


Fig. 3. Time-resolved *in situ* interaction of 5-LO and FLAP in activated monocytes and neutrophils.

In situ PLA, using proximity probes against mouse anti-5-LO and rabbit anti-FLAP, was performed in human monocytes (top) and neutrophils (bottom) upon stimulation with A23187 (2.5 µM) for the indicated time points. DAPI (blue) was used to stain the nucleus and *in situ* PLA signals (magenta dots) visualize 5-LO/FLAP interaction; scale bar = 10 µm (insets monocytes) and 15 µm (insets neutrophils). Results are representative for approx. 100 individual cells of three independent experiments.

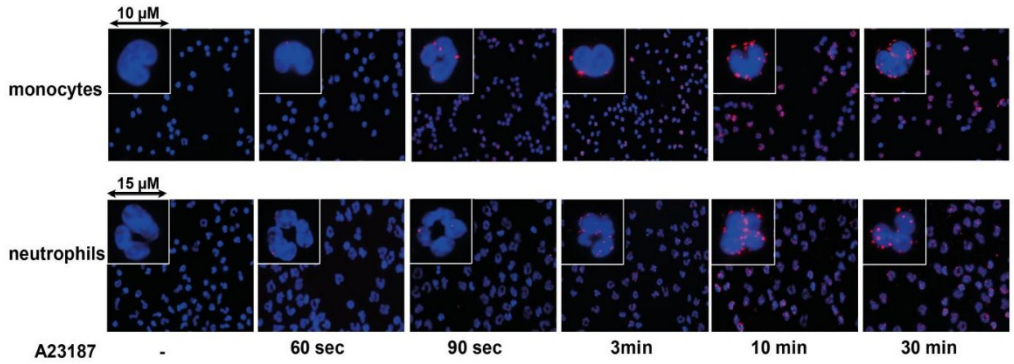
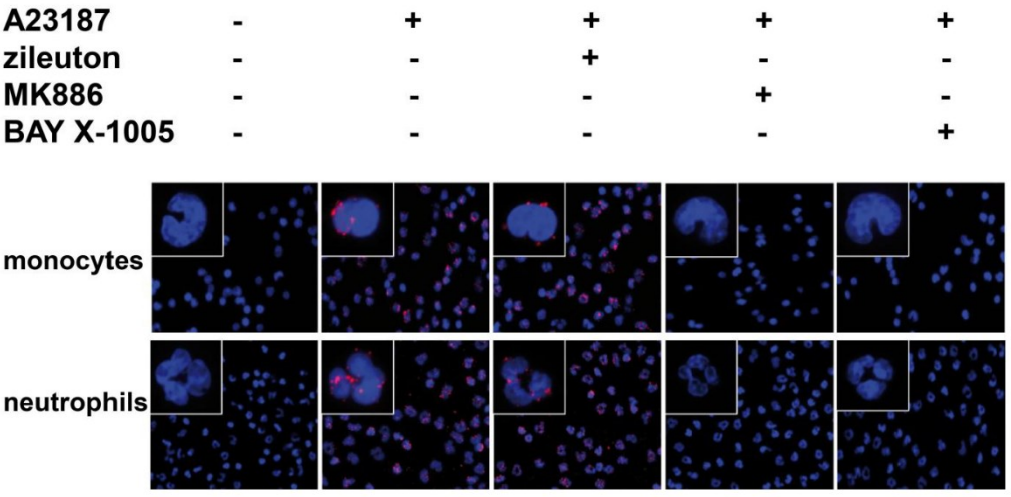


Fig. 4. FLAP inhibitors prevent 5-LO/FLAP complex formation.

Monocytes (top lane) and neutrophils (bottom lane) were exposed to the direct 5-LO inhibitor zileuton (10 µM) as well as to FLAP inhibitors MK886 (100 nM) and BAY X-1005 (3 µM) for 15 min at 37 °C, before stimulation with A23187 (2.5 µM) for 15 min at 37 °C. DAPI (blue) was used to stain the nucleus and *in situ* PLA signals (magenta dots) visualize 5-LO/FLAP interaction; scale bar = 10 µm (A) and 15 µm (B). Results are representative for approx. 100 individual cells of three independent experiments.



15

Fig. 5. Reversal of cPLA$_2$ inhibitor-induced loss of 5-LO/FLAP interaction by exogenous AA and 5-HpETE.

PLA assay was performed in human monocytes (top lane) and neutrophils (bottom lane). Cells were exposed to cPLA$_2\alpha$ inhibitor RSC-3388 (5 μM) for 15 min at 37 °C, before stimulation with 2.5 μM A23187 alone, A23187 plus 5 μM 5-HpETE or A23187 plus 10 μM AA for 30 min at 37 °C. DAPI (blue) was used to stain the nucleus and *in situ* PLA signals (magenta dots) visualize 5-LO and FLAP interaction; scale bar = 10 μm (A) and 15 μm (B). Cells were analyzed by a Zeiss Axiovert 200M microscope, and a Plan Neofluar ×100/1.30 Oil (DIC III) objective for single cell images (insets, scale bar = 10 μM and 15 μM for monocytes and neutrophils, respectively) as well as a Plan Neofluar ×40/1.30 Oil (DIC III) objective for overview images. Results are representative of three independent experiments.

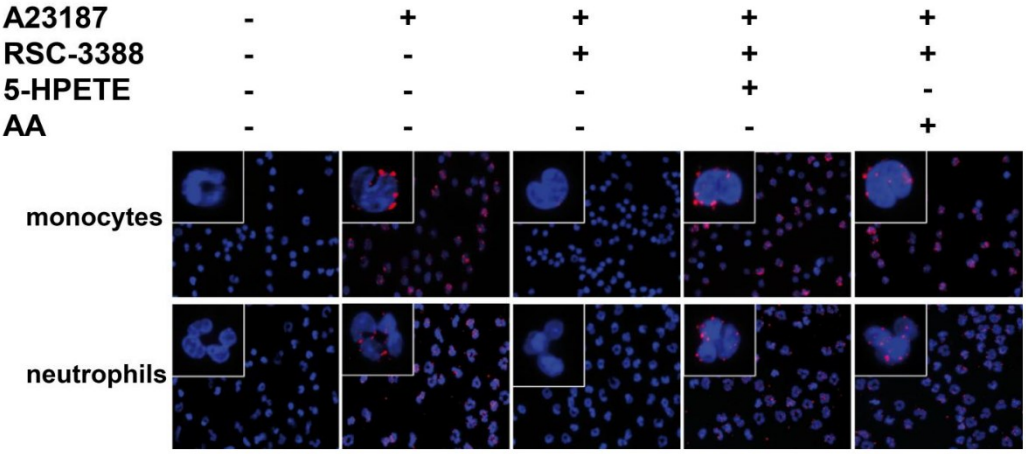
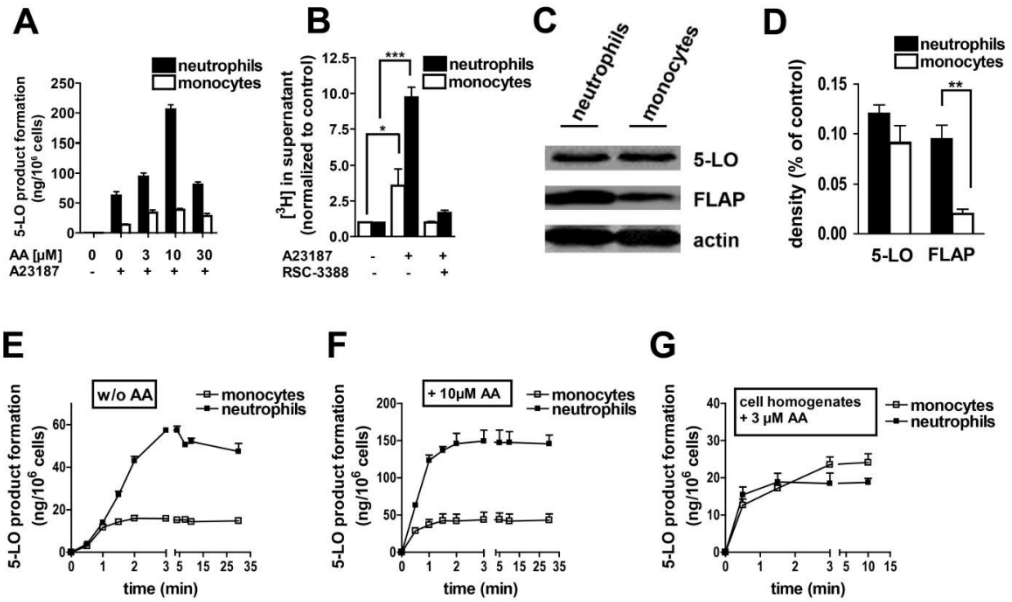


Fig. 6. Cellular 5-LO product synthesis in human monocytes and neutrophils upon A23187 activation.

(A) Amounts of 5-LO products formed in human monocytes and neutrophils after activation with 2.5 µM A23187 with or without exogenous AA for 10 min at 37 °C. (B) Analysis of released [^{3}H]AA and its metabolites in supernatants of monocytes and neutrophils, normalized to unstimulated control, after preincubation with 1 µM cPLA$_2\alpha$ inhibitor (RSC-3388) or vehicle and stimulation with 2.5 µM A23187 for 10 min. (C) Protein levels of 5-LO and FLAP in neutrophils and monocytes were analyzed by Western blotting. Densitometric analysis (D) (n=3) shows the intensities of respective bands normalized to β-actin. (E/F) Time-resolved 5-LO product formation in intact human monocytes (-□-) and neutrophils (-■-) upon stimulation with 2.5 µM A23187 (E) or 2.5 µM A23187 plus 10 µM AA (F). (G) Time-resolved 5-LO product formation in homogenates of human monocytes (-□-) and neutrophils (-■-) from 3 µM AA. 5-LO products include LTB$_4$, its all-trans isomers and 5-H(p)ETE, analyzed by RP-HPLC. Data are expressed in means ± SEM; n = 3, duplicates; ***p < 0.001, **p < 0.01, *p < 0.1; vs. unstimulated control, Student´s *t* test.



Supplementary Information

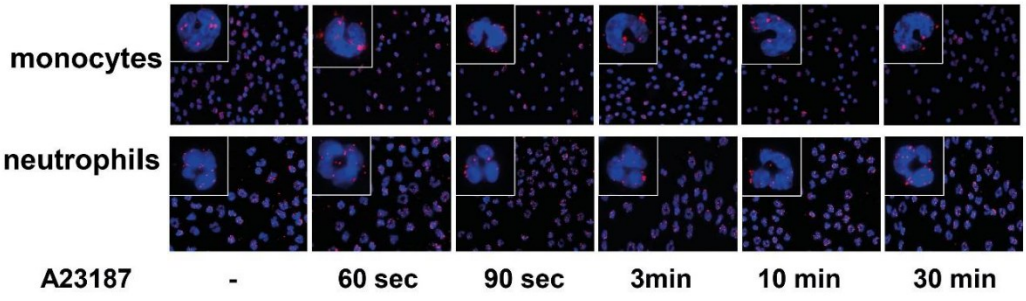
Time-resolved *in situ* assembly of the leukotriene-synthetic 5-LO / FLAP complex in blood leukocytes

Jana Gerstmeier[1], Christina Weinigel[2], Silke Rummler[2], Olof Rådmark[3], Oliver Werz[1], Ulrike Garscha[1]

Figure Legend for S1.

Time-resolved *in situ* interaction of 5-LO and CLP in activated monocytes and neutrophils. *In situ* PLA, using proximity probes against mouse anti-5-LO and rabbit anti-CLP was performed in human monocytes (top) and neutrophils (bottom) upon stimulation with A23187 (2.5 μM) for the indicated time points. DAPI (blue) was used to stain the nucleus and *in situ* PLA signals (magenta dots) visualize 5-LO/CLP interaction. Cells were analyzed by a Zeiss Axiovert 200M microscope, and a Plan Neofluar ×100/1.30 Oil (DIC III) objective for single cell images (insets, scale bar = 10 μM and 15 μM for monocytes and neutrophils, respectively) as well as a Plan Neofluar ×40/1.30 Oil (DIC III) objective for overview images. Results are representative for 100 individual cells of three independent experiments.

Fig. S1.



4. DISCUSSION

This thesis combines diverse experimental approaches to define the molecular basis for the complex assembly of 5-LO and FLAP in intact cells to produce LTs, and its pharmacological inhibition. LTs are potent lipid mediators in the pathological development of diseases like asthma, rhinitis, cancer, and CVD [3]. Upon cell activation, endogenous released AA is converted by 5-LO in a two-step reaction that proceeds via the intermediate 5-HPETE (dioxygenase activity) to LTA_4 (epoxidase activity) [12]. In contrast to a cell-free environment, the fate of 5-LO to be catalytic active *in cellulo* is strongly controlled by compartmentalization with enzymes in the cytosol, nuclear membrane, and extracellular space. The compartmentalized biosynthetic LT pathway offers opportunities to abate inflammatory responses through the dynamic control of the subcellular localization of 5-LO as key enzyme for LT biosynthesis. A fine-tuned regulatory machinery ascertains 5-LO activity, stability, localization, and protein-protein interactions, as well as 5-LO self-inactivation. Cellular 5-LO catalysis depends on an essential translocation process from the cytosol or intranuclear region to the nuclear membrane-bound FLAP upon activation [2]. 5-LO activity and access to the LT precursor AA is believed to be aided by FLAP [107]. It is assumed that FLAP acts as a scaffold for 5-LO that ascertains the association of 5-LO at the perinuclear region to enable efficient LTA_4 formation at this locale [34]. FLAP has been shown to bind AA as well as certain other fatty acids [107] but how FLAP transfers the substrate to 5-LO for efficient metabolism, facilitates 5-LO catalytic activity, and whether an interaction between the two proteins is required remain unanswered questions to date. It has still not been defined what happens throughout the catalytic cycle of 5-LO at the nuclear membrane These are important questions to address as FLAP was shown to determine the subcellular localization of 5-LO, and thus its capacity to produce LTs. Lack of FLAP or pharmacological inhibition *in cellulo* or *in vivo* completely prevents LT formation from endogenous sources of AA [4], and FLAP antagonists were reported to intervene with the essential 5-LO translocation to the perinuclear region [59, 110]. Thus, it is not surprising that the pharmacological development of compounds targeting FLAP instead of 5-LO to reduce LT biosynthesis has been constantly increased since the discovery of FLAP. Antagonists of FLAP were shown to be promising drug candidates for the treatment of inflammatory and allergic

diseases by interference with the 5-LO pathway [4], and to be highly beneficial in the treatment of CVD [21]. An additional exceptional advantage of blocking FLAP instead of 5-LO, is the recently discovered involvement of cytosolic 5-LO for the formation of pro-resolving LXA_4 [134]. Cytosolic localization of 5-LO is believed to be regulated by FLAP expression and its antagonists. Compounds targeting FLAP like MK886, MK591 or BAY X-1005 are highly efficient in limiting cellular 5-LO product formation from endogenous AA [91], but are less potent *in vivo* and eventually failed in clinical trials, Recent developments like GSK2190915 [99] solely concentrate on the classical FLAP inhibitor type MK886, whereas novel structures are rare.

4.1 Pharmacological characterization of the novel FLAP antagonist BRP-7

Encouraged by the therapeutic potential of FLAP as promising drug target, a novel chemotype for FLAP inhibitors was recently discovered by us in a virtual screening approach based on a combined ligand- and structure-based pharmacophore model (Fig. 9) [5].

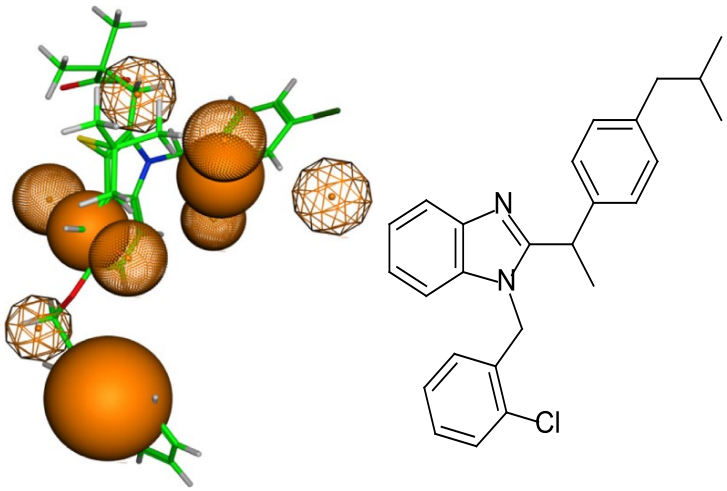


Fig. 9: Ligand- and structure-based pharmocophore model reveals novel chemotype for FLAP inhibitors. (Right) Pharmacophore model consists of three aromatic rings, three hydrophobobic features and the respective spatial arrangement. (Left) Chemical structure of BRP-7.

The innovative compound BRP-7 [1-(2-chlorobenzyl)-2-(1-(4-isobutylphenyl)ethyl)-1H-benzimidazole] potently prevented 5-LO activity in intact human leukocytes but failed to directly inhibit 5-LO, suggesting FLAP inhibition as mode of action. The structural key element of BRP-7 is a non-acidic benzimidazole scaffold with an

isobutylphenylethyl ibuprofen fingerprint. This is in sharp contrast to the well-established quinoline- (BAY X-1005) or indole-based (MK886) FLAP inhibitors that both contain a carboxylic acid group, and strongly differ in the overall structure. Thus, BRP-7 is an excellent candidate for further developments of novel innovative FLAP antagonists. Unlike others, BRP-7 not only prevented LT biosynthesis *in vitro*, but was also effective *in vivo*. BRP-7s pharmacology was intensively studied within the frame of this thesis and revealed (I) significant cellular inhibition of 5-LO activity in isolated primary human neutrophils (IC_{50}: 150 nM) and monocytes (IC_{50}: 40 nM) (II) without direct inhibition of 5-LO, (III) interference with 5-LO translocation to the nuclear membrane, (IV) specific inhibition of FLAP without side effects on other enzymes of the AA cascade, (V) no inhibition of CYP3A4 or hERG, and (VI) reduced LT biosynthesis in HWB assays, and *in vivo* animal models of acute inflammation. Together, the results provide strong evidence that the pharmacological target of BRP-7 is FLAP.

The compound exhibits typical properties of well-characterized FLAP inhibitors, that is, high cellular efficacy and poor inhibition of LTs in cell-free assays like cell homogenates or isolated recombinant 5-LO [135]. Up to 100-times higher concentrations of BRP-7 are required to inhibit 5-LO product synthesis under cell-free conditions which is similar to the pharmacology of MK886. Of interest, non-redox type 5-LO inhibitors act similar in cell-free assays due to a lack of reducing agents (e.g. GSH) [47]. However, BRP-7 did not inhibit 5-LO activity in leukocyte homogenates upon supplementation with GSH, which excludes a function as non-redox type 5-LO inhibitor but supports the hypothesis of FLAP as cellular target for BRP-7. Exogenous substrate supply has been shown to evade FLAP as AA transfer protein [4]. As expected, high excess of free AA to A23187-stimulated intact leukocytes abated BRP-7`s potency in a concentration-dependent manner. Seemingly, BRP-7 competes with AA for the binding to FLAP as reported before for other FLAP antagonist [100].

To confirm FLAP as the proposed target, the compound was coupled to an affinity matrix and isolated nuclear membranes from human neutrophils were used as sources of FLAP for *in vitro* pull-down assays. Indeed, FLAP was enriched in pull-downs of matrix-coupled BRP-7 which was competitively abolished by high excess of soluble BRP-7. In sharp contrast to MK886, no intervention with other AA-metabolizing

enzymes like COX-1, 12- or 15-LO, and mPGES-1 or AA-releasing $cPLA_2$-α was evident, confirming BRP-7s high selectivity for FLAP.

Although FLAP inhibitors were reported to impair the translocation of soluble 5-LO to the nuclear membrane for the association with FLAP [79], only high concentrations of BRP-7 marginally reduced the 5-LO/FLAP co-localization. This indicates that LT suppression by BRP-7 is not directly related to an impaired 5-LO translocation and co-localization with FLAP. Interestingly, BRP-7 reduced LT biosynthesis more prominent in monocytes (IC_{50}: 40 nM) than in neutrophils (IC_{50}: 150 nM), which might be related to diverse functional relevancies of FLAP for cellular LT biosynthesis in neutrophils versus monocytes.

Unlike other FLAP inhibitors, BRP-7 remained highly effective in HWB assays and two well-established *in vivo* animal models of acute inflammation: carrageenan-induced pleurisy in rats and zymosan-induced peritonitis in mice [104]. A major drawback of previous FLAP inhibitor studies has been their strong interference with CYP enzymes [123], but no significant inhibition of CYP3A4 by BRP-7 was evident. Also, neither hERG inhibition nor toxic effects on cell integrity and viability were observed for BRP-7-treated mice and rats. Together, the detailed *in vitro* and *in vivo* study of BRP-7 suggests FLAP as its pharmacological target, and excludes direct inhibition of 5-LO, $cPLA_2$-α or other AA-metabolizing enzymes. However, no straight forward and smart model was available at this time to determine a direct functional interference of BRP-7 with FLAP. Certainly, it is challenging to study the functionality of FLAP and its relevance for LT biosynthesis as FLAP is devoid of enzymatic activity and the 5-LO/FLAP interaction is limited to the cellular context. Also, FLAP is an integral membrane protein that needs a bilayer surrounding to express full functionality, limiting studies with the purified protein [125].

To date, putative antagonist of FLAP have been evaluated for their efficiency to suppress 5-LO activity solely in intact cells but failed to inhibit in cell-free assays as discussed for BRP-7, or radioactive-labelled inhibitor analogues were used to determine their binding affinity towards FLAP from nuclear membrane preparations of human leukocytes [95]. For this assay however, competitive binding of the inhibitor with AA to FLAP is essential, whereas allosteric regulators cannot be distinguished.

Major drawbacks of previous attempts to establish a cell-based model to identify putative FLAP antagonists and investigate the assembly of the LT biosynthetic complex at the nuclear membrane in mammalian cells were the use of (I) transiently expression systems [2, 122] and (II) artificial (cell-free) GFP- or GST-tagged 5-LO or FLAP fusion proteins [136] that eventually interfere with the biological relevance and reproducibility of the experiments. However, there is strong need for an improved knowledge about the assembly of the 5-LO/FLAP interaction as a better understanding of this biosynthetic complex at the nuclear membrane and its functionality will help to develop novel potent FLAP inhibitors with multi-points of attack, and expedite pharmacological intervention strategies for improved therapeutic approaches in LT-associated diseases.

4.2 FLAP promotes cellular LTA_4 synthase activity of 5-LO

The molecular and mechanistic basis of the 5-LO/FLAP interaction has been elusive as FLAP modulates 5-LO activity only at the cellular level and could not yet be recapitulated *in vitro*.Thus, the next key step of this thesis was the development of an experimental cell-based system to study the *in cellulo* 5-LO/FLAP interaction, elucidate FLAP´s functionality in LT biosynthesis, and establish a suitable tool to screen for novel FLAP inhibitors. In brief, HEK293 were stably transfected with 5-LO alone (HEK-5-LO) or together with FLAP (HEK-5-LO/FLAP). Unlike previous studies with isolated human primary leukocytes or mammalian cell lines that express endogenous 5-LO and FLAP, the HEK293 cell system allows to obtain deeper insights into the assembly of the putative 5-LO/FLAP complex for example by considering site-directed mutagenesis experiments. Promising residues for future investigations in helix α2 of human 5-LO are Phe^{177} and Tyr^{181}, often referred to as the “FY-cork”, that appear to control the entrance of AA to 5-LO`s active site [27], or deletions of residues within the two cytosolic loops of FLAP that are exposed to the membrane, and thus regions most likely to interact with 5-LO [100]. Equal expression levels of FLAP and 5-LO in the HEK293 cells were verified by immunoblotting. 5-LO product formation was analyzed

by HPLC in intact cells versus cell homogenates, and the subcellular localization of the proteins was determined by IF microscopy.

Both cell lines efficiently metabolized exogenous AA upon stimulation with the Ca^{2+}-mobilizing agent A23187. Detectable 5-LO products comprised 5-H(p)ETE and the trans-isomers of LTB_4. As FLAP is supposed to function as a transfer protein for endogenously released AA to 5-LO [107], we expected significant amounts of LTs upon activation by A23187 without supply of exogenous substrate in cells co-expressing FLAP. Unexpectedly, no detectable amounts of 5-LO products (<2 ng/10^6 cells) in HEK-5-LO/FLAP as well as HEK-5-LO cells were observed after stimulation with A23187 for 10 min at 37 °C. Control experiments with primary human neutrophils and monocytes under equal assay conditions led to significant amounts of LTs as reported before [17, 83]. Moreover, a stable cell line expressing platelet-type 12-LO (HEK-12-LO) generated 12-H(p)ETE after activation by A23187, which is in sharp contrast to 5-LO expressing cells, regardless of FLAP. 12-H(p)ETE was consistently produced for more than 15 min after an unusual lag phase of about 2 min. In human platelets however, 12-LO metabolizes endogenous released AA within seconds upon stimulation [102] which indicates an impaired AA release in HEK293 cells compared to primary blood cells. Although 5-LO in cells with and without FLAP failed to produce LTs from endogenous sources of AA, the addition of 3, 10 or 30 μM AA to A23187-stimulated cells led to significant amounts of 5-LO products, superior to those observed in human primary monocytes and neutrophils in parallel experiments.

The unusual lag phase in HEK-12-LO cells to form AA-derived products and the low LT formation from endogenous AA in 5-LO and FLAP co-expressing cells prompted us to investigate the $cPLA_2$-α-mediated AA hydrolysis from phospholipids within the membrane. Potentially, $cPLA_2$-α is not as functional active in HEK293 cells as it is in human leukocytes to evoke the complex assembly of 5-LO and FLAP. However, ^{3}[H]AA-labelled HEK293 cells released significant amounts of AA-derived products upon activation with A23187 that could be inhibited by the specific $cPLA_2$-α inhibitor RSC-3388. These results preclude an absolute lack of liberated endogenous AA in A23187-stimulated HEK293 cells. Compared to human neutrophils and monocytes however, the relative amount of liberated AA in HEK293 cells was rather weak and delayed, most likely insufficient to enable the complex assembly of 5-LO and FLAP at

the nuclear membrane but appears to be sufficient for cytosolic 12-LO activity (HEK-12-LO). Also, we cannot exclude that in HEK293 cells AA gets liberated from other lipid sources than the nuclear membrane, as cPLA$_2$-α has been shown to translocate also to the endoplasmic reticulum, as well as Golgi apparatus [113]. In fact, AA release from these membranes might be accessible for cytosolic 12-LO but not for nuclear membrane-associated 5-LO. Finally, the content of phospholipids containing sufficient AA in sn-2 position may be limited in HEK293 cell cultures versus primary neutrophils or monocytes.

It appeared possible that 5-LO/FLAP co-localization upon translocation of soluble 5-LO is not operative in the HEK cell lines. Thus, the subcellular localization of 5-LO and FLAP in resting and activated cells was analyzed by IF microscopy. Unstimulated cells revealed a rather homogenous distribution of soluble 5-LO in the nucleus and in the cytosol. In cells lacking FLAP, 5-LO essentially redistributed in the intranuclear region without marked nuclear membrane association upon stimulation. This is in sharp contrast to FLAP co-expressing cells. Here, soluble 5-LO translocated to the nuclear membrane, harbouring FLAP. Both proteins clearly co-localized at this locale. This co-localization was impaired by high concentrations of the FLAP antagonist MK886 (300 nM), which is in agreement with reported MK886 properties in human leukocytes [110]. The subcellular localization of 5-LO in MK886-treated HEK-5-LO/FLAP cells resembled a similar pattern observed in A23187-activated cells lacking FLAP.

An improved membrane binding of 5-LO is considered to promote LTA$_4$ biosynthesis [29], but how FLAP facilitates the efficient metabolism of AA remained elusive. In fact, FLAP co-expression significantly promoted the conversion of exogenous AA to increased levels of LTA$_4$ compared to cells lacking FLAP. In particular, the formation of LTA$_4$ at 3 µM AA, resembling the physiological concentration of liberated AA of A23187-stimulated primary human leukocytes [103], was significantly pronounced in HEK-5-LO/FLAP cells. The ratio of 5-H(p)ETE versus trans-isomers of LTB$_4$ was markedly reduced from 6:1 in HEK-5-LO to 2:1 in HEK-5-LO/FLAP cells. The observed FLAP-mediated effect was blocked by MK886 in a concentration-dependent manner. This is of interest as MK886 was shown to compete with AA for the binding pocket in FLAP [95]. Under conditions where FLAP supported 5-LO activity, MK886 reduced LT biosynthesis by 35% at low nanomolar concentrations. In fact, predominantly the

formation of LTA_4 was blocked by MK886, and thus the described 5-LO-stimulatory effect of FLAP. No similar observations were made for the direct 5-LO inhibitor zileuton. As expected, MK886 did not reduce LT biosynthesis in intact HEK-5-LO cells lacking FLAP, and was ineffective in homogenate preparations of both cell lines independently of FLAP expression. Note that FLAP is dispensable for 5-LO activity under cell-free assay conditions [137]. The direct 5-LO inhibitor zileuton however, equally inhibited 5-LO activity in intact cells and homogenates, regardless of the presence of FLAP. As described before for several FLAP inhibitor studies in human leukocytes including BRP-7, substantial amounts of exogenous AA (e.g. 30 µM) circumvent the necessity of FLAP as substrate transfer protein for 5-LO in intact cells [138], and all described unique characteristic of FLAP co-expressing cells were vanished. No significant increase of 5-LO activity nor differences in the formed metabolite profile were determined for both cell lines. Together, our results highlight FLAPs central role as 5-LO-stimulating protein in intact cells for *in situ* LT biosynthesis.

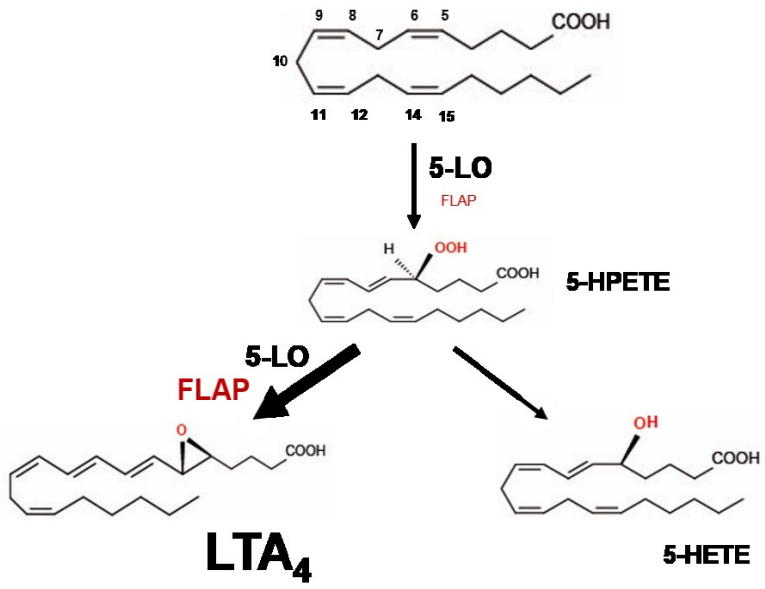


Fig. 10: FLAP controls the conversion of AA to LTA_4. 5-HPETE formation from AA by 5-LO appears to be largely independent of FLAP co-expression, whereas efficient LTA_4 formation is prompted in the presence of FLAP.

To date, FLAP has been known to function as a transfer protein for endogenous released AA, possibly anchoring 5-LO to the nuclear membrane for substrate acquisition [34, 139]. Our data provide strong evidence that FLAP not only transfers AA to 5-LO but also positions the intermediate 5-HPETE at the active site to assure efficient LTA_4 formation. Hence, we expose an additional function of FLAP, that is,

support of the LTA_4 synthase activity of 5-LO without marked effects on the 5-HETE production (Fig. 10).

The established HEK293 cell model is a suitable tool to study the role of FLAP for 5-LO catalysis, and to distinguish putative FLAP antagonists from interference with 5-LO. In fact, FLAP inhibitors are highly beneficial for the treatment of LT-associated diseases in current clinical trials [134], and the described cellular model may contribute to identify highly potent FLAP antagonists in early preclinical studies. Although FLAP and 5-LO co-localized at the nuclear membrane, and endogenous AA was liberated by $cPLA_2$-α, no 5-LO products were detectable. Upon exogenous supply of AA however, LT biosynthesis was significantly increased in the presence of FLAP. In conclusion, other enzymes of the AA cascade like $cPLA_2$-α, LTC_4S and/or LTA_4H might be involved in the assembly of the fine-tuned LT biosynthetic complex, which is functional in primary human cells but not completely operative in the established cell model. Hence, further studies in primary cells appear necessary for a complete understanding of the fine-tuned machinery of the 5-LO/FLAP pathway.

4.3 Time-resolved *in situ* interaction of native 5-LO and FLAP

Biochemical and IF microscopy data clearly pointed out a strong need for 5-LO to translocate to the nuclear membrane-embedded FLAP in intact cells in order to gain full activity, but do they really physically interact? Questions regarding the often proposed interaction between 5-LO and FLAP for cellular LT biosynthesis remain unanswered, and if additional factors regulate this interaction in intact human primary cells could not be addressed with the established HEK293 cell model described above. Previous attempts to reveal the mechanism for the tight assembly of the 5-LO/FLAP complex at the nuclear membrane were rather unsatisfying. Major drawbacks of previous studies were the use of artificial fusion-proteins instead of native 5-LO and FLAP in other species than human. Despite *in vitro* pull-down assays and co-immunoprecipitation experiments with GST-tagged 5-LO and FLAP [106, 111], or GFP-based fluorescence lifetime imaging microscopy (FLIM) analysis for association

studies upon enforced protein cross-linking [106], no persuasive proof for a direct and effective interaction of native 5-LO and FLAP in primary human leukocytes has been provided, until now. Moreover, the subcellular co-localization of two proteins, visualized by IF microscopy, does not necessarily imply a tight and functional active protein-protein interaction.

For the first time, we determined the time-resolved subcellular localization of 5-LO, concomitant with LT biosynthesis, and provide strong evidence for an effective *in situ* interaction of native 5-LO and FLAP at the nuclear membrane in A23187-activated primary human leukocytes and stable transfected HEK293 cells by proximity ligation assay (PLA). What is particular attractive regarding this sophisticated method is the possibility to detect *in situ* protein-protein interactions of unmodified, native proteins (no tags, dyes, or labels) at a defined *cellular* locale [6]. A PLA signal is detectable by fluorescence microscopy if the two proteins of interest are in close proximity with a distance < 40 nm.

Among all 5-LO and FLAP expressing mature myeloid cells [30], monocytes and neutrophils possess the highest capacity to generate LTs [140, 141]. At first, we compared the amounts of produced LTs from both cell types in intact cells and homogenates upon stimulation. Interestingly, intact neutrophils metabolized 3 to 5-times higher 5-LO products versus intact monocytes, but followed a similar kinetic course in crude cell homogenates with equivalent levels of produced LTs. This implies a similar catalytic active 5-LO in both cell types per se. Note that 5-LO activity after cell homogenization is mostly independent of cell type-specific regulation [137], and FLAP supports 5-LO activity solely *in cellulo* [34]. This was already demonstrated in the HEK293 cell model and pharmacological study of BRP-7. In line with these findings, equal protein expression levels of 5-LO in neutrophils and monocytes were verified by Western Blot, normalized to actin. The expression level of FLAP however, was significantly lower in monocytes. This is of interest as FLAP is supposed to facilitate the transfer of endogenously released AA to 5-LO [107]. It is tempting to consider the higher expression level of FLAP in neutrophils as reason for the improved product formation. Also, it appeared possible that the observed differences in LT biosynthesis may also result from an enhanced AA liberation by $cPLA_2$-α. Indeed, A23187-stimulated neutrophils released significantly higher amounts of AA and its metabolites

from [^{3}H]AA-labelled neutrophils compared to monocytes which could be blocked by the cPLA$_2$-α inhibitor RSC-3388. To avoid cell-type specific discrepancies in the cPLA$_2$-α activity, increasing concentrations of exogenous AA (3, 10 or 30 µM) were added to A23187-stimulated neutrophils and monocytes.To our surprise, LT biosynthesis was again 3- to 5-times more pronounced in neutrophils versus monocytes, independent of exogenous substrate supply. Consequently, the superior 5-LO product formation in neutrophils cannot simply be explained by an improved AA liberation rate which prompted us to analyse the LT biosynthesis from endogenous sources of AA in a time-dependent manner. Indeed, a rather delayed and significantly prolonged 5-LO activity in neutrophils compared to monocytes was observed. While LT biosynthesis in monocytes was essentially completed 60-90 sec post stimulation without significant differences in the kinetic course of 5-LO product formation in neutrophils at this time, 5-LO catalysis in neutrophils continued up to 180 sec, resulting in higher levels of LTs. Nevertheless, the impact of a more physiological relevant stimulus like LPS plus fMLP or PAF should be addressed in future studies as the immediate and receptor-independent Ca^{2+} ion influx in A23187-stimulated cells circumvents complex signalling pathways that may account for cell-type specific regulation of 5-LO in monocytes and neutrophils [121].

As discussed above, 5-LO catalysis depends on an essential translocation process during activation to establish the LTA$_4$-biosynthetic complex with FLAP at the nuclear envelope. Therefore, we monitored the time-dependent subcellular localization of 5-LO and FLAP by IF microscopy. This is in sharp contrast to previous studies that determined the 5-LO/FLAP co-localization solely after one fixed time-point (10 or 30 min) post stimulation [106, 120]. Our kinetic analysis of LT biosynthesis however, indicated a limited period of active 5-LO within the first 3 min upon activation. Again, 5-LO nuclear membrane association and co-localization with FLAP was more rapid in monocytes compared to neutrophils, and appeared earliest at 60-90 sec upon stimulation without alterations up to 30 min. In line with prolonged LT biosynthesis in neutrophils, 5-LO translocation occurred delayed and continued up to 180 sec. Thus, 5-LO translocation strongly correlated with the time periods for 5-LO product formation. In conclusion, 5-LO catalysis occurs as long as a pool of soluble 5-LO traffics to the nuclear membrane-bound FLAP for substrate acquisition and turn-over. In the cell, 5-

LO activity appears to be terminated by membrane and/or FLAP association, leading to irreversible turnover-based suicide inactivation of 5-LO [89, 90]. Similar observations were made for purified 5-LO in the presence of AA and PC vesicles. The rate of inactivation primarily depends on substrate accessibility and concentration, as well as lipid peroxides within the phospholipid membrane [88], eventually terminating 5-LO product synthesis by non-turnover inactivation. Together, the data indicate that enhanced LT biosynthesis in neutrophils is a result of a prolonged translocation pool of active 5-LO. How membrane and/or FLAP association however, leads to 5-LO inactivation remained elusive. IF microscopy of 5-LO and FLAP allowed only predictions about their co-localization at a defined locale without proof for a direct and physical protein-protein interaction.

To investigate the tight assembly of 5-LO and FLAP, a novel technique for the detection of direct protein-protein interactions was applied, the PLA. Unlike GFP-based FRET approaches, PLA probes capture the interacting proteins in their native form (Fig. 11). This is especially important for the 5-LO/FLAP interaction, which may be transient with a lifetime of a single catalytic cycle.

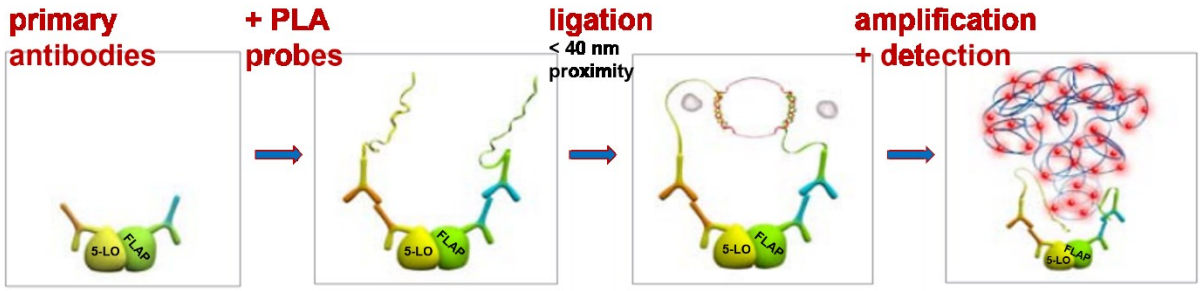


Fig. 11: Principle of *in situ* PLA. Cells were incubated with primary antibodies against 5-LO (mouse) and FLAP (rabbit), incubated with PLA probes anti-mouse PLUS and anti-rabbit MINUS, ligated to a closed circle < 40 nm proximity, followed by amplification and fluorophore hybridization (magenta dots).

As expected, 5-LO and FLAP do not interact in resting cells due to different distributions in the cell. Upon A23187-stimulation, 5-LO traffics to the nuclear membrane harbouring FLAP, and a clear PLA signal illustrating the 5-LO/FLAP interaction was detected in human primary leukocytes as well as stable transfected HEK293 cells. Note that the

PLA signal appeared earliest around 90 sec in monocytes and not until 3 min post stimulation in neutrophils, time-points where LT biosynthesis and 5-LO translocation was already completed. Interestingly, the signal increased up to 30 min post activation in both cell-types. We are aware of the possibility that AA may be required for such an interaction, and speculate that 5-LO first loosely associates with FLAP upon translocation for substrate acquisition and turn-over before a tight interaction occurs. As long as LT biosynthesis proceeds, both proteins are not yet within the distance for ligation of the PLA probes. Consequently, the 5-LO/FLAP interaction occurs delayed compared to 5-LO translocation and product formation. The data indicate that FLAP not simply functions as membrane anchor for 5-LO but also brings the enzyme in a resting mode upon completed catalytic cycle to temper LT biosynthesis.

In sharp contrast to the stimulation-dependent 5-LO/FLAP assembly, 5-LO interacts permanently and independent of stimuli with its scaffold CLP, which was determined before by co-IP [59]. Monitoring the 5-LO/CLP interaction resulted in a strong PLA signal without time-related discrepancies in monocytes and neutrophils. Although previous studies suggested a pronounced association of CLP with 5-LO upon Ca^{2+}-binding [33, 59], the PLA signal did not increase upon A23187-mediated Ca^{2+} influx. However, a Ca^{2+}-dependent binding mode might not be detectable by PLA but could influence the extent of co-IP experiments.

To further validate and characterize the 5-LO/FLAP assembly, HEK293 cells stably expressing 5-LO with or without FLAP were analyzed. As expected, PLA signals were only evident in A23187-stimulated FLAP co-expressing cells. Second, when HEK293 cells were transfected with the 5-LO_3W mutant ($Trp^{13/75/102}Ala$), unable to associate with the nuclear membrane and co-localize with FLAP in IF experiments, no PLA signal was detected independent of stimulation and FLAP co-expression. An abolished binding to PC [41] and/or CLP by the 5-LO_3W mutant [33] is most likely the reason for the observed insufficient translocation process and association with FLAP.

Finally, FLAP inhibitors are known to compete with AA for the binding site at FLAP, and to interfere with FLAP`s function as transfer protein for AA to activated and membrane-associated 5-LO which prompted us to investigate the influence of two structural unrelated FLAP inhibitors MK886 [110] and BAY X-1005 [92] on the assembly of 5-LO

and FLAP. Both FLAP antagonists did not impair the 5-LO translocation and co-localization with FLAP, but the PLA signal was clearly abolished upon inhibitor treatment prior stimulation. The direct 5-LO inhibitor zileuton [142] did not influence the 5-LO/FLAP co-localization and interaction. Consequently, future inhibitor studies need to distinguish between 5-LO/FLAP co-localization and 5-LO/FLAP interaction.

In the HEK293 cell model FLAP not only promoted substrate acquisition and conversion of AA to 5-HPETE but also the dehydration of 5-HPETE to LTA_4. To investigate the regulating function of AA and 5-HPETE for the 5-LO/FLAP interaction, AA release was inhibited by the $cPLA_2$-α inhibitor RSC-3388 prior stimulation. Again the assembly of the 5-LO/FLAP complex was reduced, whereas 5-LO translocation and co-localization with FLAP was not affected as reported by others before [120]. Of interest, exogenous supply of AA (10 µM) and 5-HPETE (5 µM) restored the complex assembly. Thus, we confirmed that AA and/or 5-HPETE possess a regulating function for the observed interaction or might induce a conformational shift of FLAP ready to interact with 5-LO. Together, competitive inhibition of AA-binding to FLAP as well as blockade of AA release prevented the interaction of 5-LO and FLAP at the nuclear membrane of activated leukocytes and HEK-5-LO/FLAP cells.

The results of this thesis highlight FLAPs role as transfer protein for AA, and support FLAPs function for 5-HPETE transfer and/or retention at 5-LOs active site to produce LTA_4. In addition to the effects of AA in our experiments, the prevention of the LTA_4-biosynthetic 5-LO/FLAP complex by FLAP inhibitors strengthens the biological relevance of our findings. We emphasize that 5-LO translocation is independent of FLAP expression or inhibition, and endogenous substrate supply by $cPLA_2$-α which is in line with an unimpaired 5-LO translocation to the perinuclear region in HEK-5-LO cells lacking FLAP. Finally, FLAP appears to be more than purely an “activating” protein for 5-LO catalysis during inflammation. The study contributes to a better understanding of the role of FLAP as regulating protein for 5-LO activity and LT biosynthesis. FLAP not simply functions as a membrane anchor for 5-LO, it appears to generate a time-dependent assembly of a loose LTA_4-biosynthetic 5-LO/FLAP complex at the nuclear membrane. At first, FLAP enables and regulates LT formation in order to eventually temper the pro-inflammatory response by retaining 5-LO at the nuclear membrane within a tight complex. It is shown for the first time that the subcellular

compartmentalization of 5-LO, the efficacy and extend to produce LTs, is highly dependent on the presence of the nuclear membrane-embedded 5-LO helper-protein FLAP (Fig. 12). The improved knowledge and established assays may help to develop and characterize innovative FLAP inhibitors, and expedite novel pharmacological intervention strategies for LT-associated diseases.

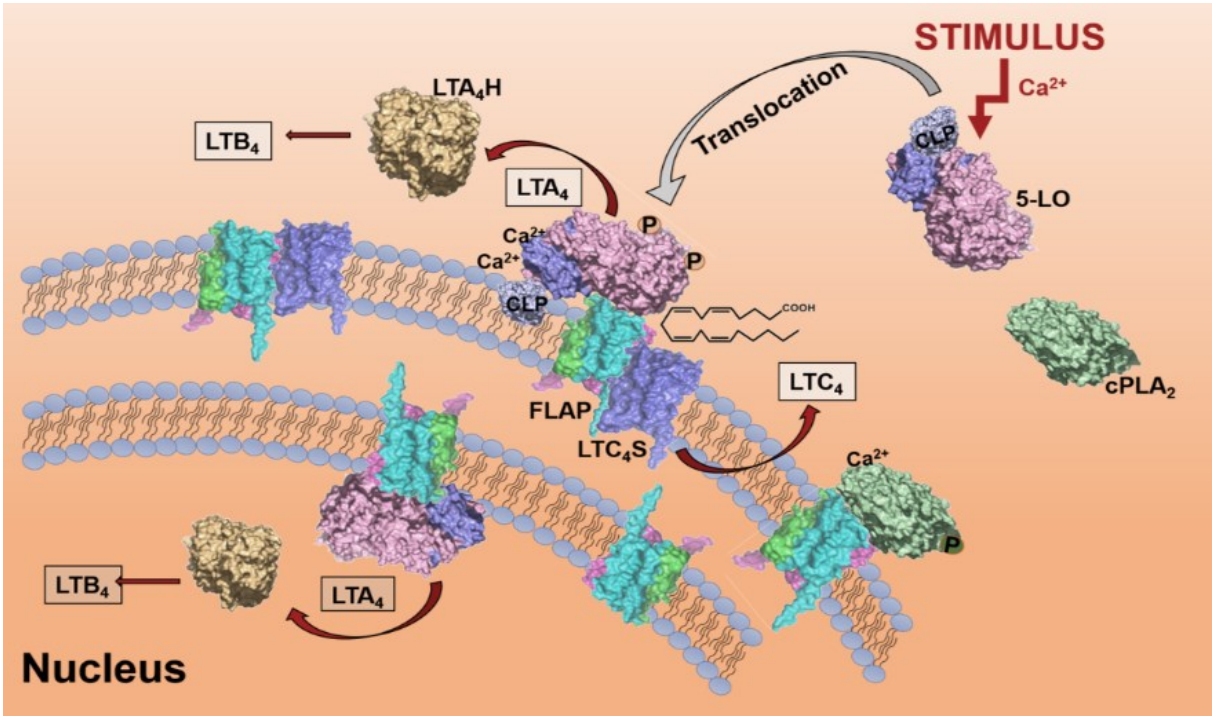


Fig. 12: The complex orchestra of LT biosynthesis in the cell. 5-LO, associated with CLP, translocates to the nuclear membrane upon stimulation. AA is released by $cPLA_2$-α, and transferred via FLAP to 5-LO for LTA_4 biosynthesis. AA and 5-HPETE regulate the assembly of 5-LO/FLAP complexes. Two populations of 5-LO/FLAP complexes are assumed to control the fate of LTA_4. Complexes at the outer nuclear membrane appear to be dedicated to LTC_4 formation by LTC_4S, while complexes at the inner membrane pronounce LTB_4 biosynthesis by LTA_4H.

5. REFERENCES

1. Samuelsson, B. (1983) Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science* **220**, 568-575

2. Dixon, R. A., Diehl, R. E., Opas, E., Rands, E., Vickers, P. J., Evans, J. F., Gillard, J. W., and Miller, D. K. (1990) Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis. *Nature* **343**, 282-284

3. Peters-Golden, M., and Henderson, W. R., Jr. (2007) Leukotrienes. *The New England journal of medicine* **357**, 1841-1854

4. Evans, J. F., Ferguson, A. D., Mosley, R. T., and Hutchinson, J. H. (2008) What's all the FLAP about?: 5-lipoxygenase-activating protein inhibitors for inflammatory diseases. *Trends in pharmacological sciences* **29**, 72-78

5. Banoglu, E., Caliskan, B., Luderer, S., Eren, G., Ozkan, Y., Altenhofen, W., Weinigel, C., Barz, D., Gerstmeier, J., Pergola, C., and Werz, O. (2012) Identification of novel benzimidazole derivatives as inhibitors of leukotriene biosynthesis by virtual screening targeting 5-lipoxygenase-activating protein (FLAP). *Bioorganic & medicinal chemistry* **20**, 3728-3741

6. Soderberg, O., Gullberg, M., Jarvius, M., Ridderstrale, K., Leuchowius, K. J., Jarvius, J., Wester, K., Hydbring, P., Bahram, F., Larsson, L. G., and Landegren, U. (2006) Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nature methods* **3**, 995-1000

7. Back, M., Sultan, A., Ovchinnikova, O., and Hansson, G. K. (2007) 5-Lipoxygenase-activating protein: a potential link between innate and adaptive immunity in atherosclerosis and adipose tissue inflammation. *Circulation research* **100**, 946-949

8. Martin, P., and Leibovich, S. J. (2005) Inflammatory cells during wound repair: the good, the bad and the ugly. *Trends in cell biology* **15**, 599-607

9. Wang, D., and Dubois, R. N. (2010) Eicosanoids and cancer. *Nature reviews. Cancer* **10**, 181-193

10. Parthasarathy, S., Wieland, E., and Steinberg, D. (1989) A role for endothelial cell lipoxygenase in the oxidative modification of low density lipoprotein. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 1046-1050

11. Lipsky, P. E. (1999) Role of cyclooxygenase-1 and -2 in health and disease. *American journal of orthopedics* **28**, 8-12

12. Panossian, A., Hamberg, M., and Samuelsson, B. (1982) On the mechanism of biosynthesis of leukotrienes and related compounds. *FEBS letters* **150**, 511-513

13. Inceoglu, B., Schmelzer, K. R., Morisseau, C., Jinks, S. L., and Hammock, B. D. (2007) Soluble epoxide hydrolase inhibition reveals novel biological functions of epoxyeicosatrienoic acids (EETs). *Prostaglandins & other lipid mediators* **82**, 42-49

14. Kuhn, H. (2000) Structural basis for the positional specificity of lipoxygenases. *Prostaglandins & other lipid mediators* **62**, 255-270

15. Samuelsson, B. (1983) Leukotrienes: a new class of mediators of immediate hypersensitivity reactions and inflammation. *Advances in prostaglandin, thromboxane, and leukotriene research* **11**, 1-13

16. Borgeat, P., Hamberg, M., and Samuelsson, B. (1976) Transformation of arachidonic acid and homo-gamma-linolenic acid by rabbit polymorphonuclear leukocytes. Monohydroxy acids from novel lipoxygenases. *The Journal of biological chemistry* **251**, 7816-7820

17. Borgeat, P., and Samuelsson, B. (1979) Arachidonic acid metabolism in polymorphonuclear leukocytes: effects of ionophore A23187. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 2148-2152

18. Borgeat, P., and Samuelsson, B. (1979) Arachidonic acid metabolism in polymorphonuclear leukocytes: unstable intermediate in formation of dihydroxy acids. *Proc Natl Acad Sci U S A* **76**, 3213-3217

19. Feldberg, W., and Kellaway, C. H. (1938) Liberation of histamine and formation of lysocithin-like substances by cobra venom. *The Journal of physiology* **94**, 187-226

20. Werz, O. (2002) 5-lipoxygenase: cellular biology and molecular pharmacology. *Current drug targets. Inflammation and allergy* **1**, 23-44

21. Poeckel, D., and Funk, C. D. (2010) The 5-lipoxygenase/leukotriene pathway in preclinical models of cardiovascular disease. *Cardiovascular research* **86**, 243-253

22. Massoumi, R., and Sjolander, A. (2007) The role of leukotriene receptor signaling in inflammation and cancer. *TheScientificWorldJournal* **7**, 1413-1421

23. Levy, B. D., Vachier, I., and Serhan, C. N. (2012) Resolution of inflammation in asthma. *Clinics in chest medicine* **33**, 559-570

24. Serhan, C. N., Hamberg, M., and Samuelsson, B. (1984) Lipoxins: novel series of biologically active compounds formed from arachidonic acid in human

leukocytes. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 5335-5339

25. Percival, M. D. (1991) Human 5-lipoxygenase contains an essential iron. *The Journal of biological chemistry* **266**, 10058-10061

26. Chasteen, N. D., Grady, J. K., Skorey, K. I., Neden, K. J., Riendeau, D., and Percival, M. D. (1993) Characterization of the non-heme iron center of human 5-lipoxygenase by electron paramagnetic resonance, fluorescence, and ultraviolet-visible spectroscopy: redox cycling between ferrous and ferric states. *Biochemistry* **32**, 9763-9771

27. Gilbert, N. C., Bartlett, S. G., Waight, M. T., Neau, D. B., Boeglin, W. E., Brash, A. R., and Newcomer, M. E. (2011) The structure of human 5-lipoxygenase. *Science* **331**, 217-219

28. Maas, R. L., Ingram, C. D., Taber, D. F., Oates, J. A., and Brash, A. R. (1982) Stereospecific removal of the DR hydrogen atom at the 10-carbon of arachidonic acid in the biosynthesis of leukotriene A4 by human leukocytes. *The Journal of biological chemistry* **257**, 13515-13519

29. Hill, E., Maclouf, J., Murphy, R. C., and Henson, P. M. (1992) Reversible membrane association of neutrophil 5-lipoxygenase is accompanied by retention of activity and a change in substrate specificity. *The Journal of biological chemistry* **267**, 22048-22053

30. Radmark, O., Werz, O., Steinhilber, D., and Samuelsson, B. (2007) 5-Lipoxygenase: regulation of expression and enzyme activity. *Trends in biochemical sciences* **32**, 332-341

31. Wiseman, J. S., Skoog, M. T., Nichols, J. S., and Harrison, B. L. (1987) Kinetics of leukotriene A4 synthesis by 5-lipoxygenase from rat polymorphonuclear leukocytes. *Biochemistry* **26**, 5684-5689

32. Noguchi, M., Miyano, M., Matsumoto, T., and Noma, M. (1994) Human 5-lipoxygenase associates with phosphatidylcholine liposomes and modulates LTA4 synthetase activity. *Biochimica et biophysica acta* **1215**, 300-306

33. Rakonjac, M., Fischer, L., Provost, P., Werz, O., Steinhilber, D., Samuelsson, B., and Radmark, O. (2006) Coactosin-like protein supports 5-lipoxygenase enzyme activity and up-regulates leukotriene A4 production. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 13150-13155

34. Abramovitz, M., Wong, E., Cox, M. E., Richardson, C. D., Li, C., and Vickers, P. J. (1993) 5-lipoxygenase-activating protein stimulates the utilization of arachidonic acid by 5-lipoxygenase. *European journal of biochemistry / FEBS* **215**, 105-111

35. Radmark, O. P. (2000) The molecular biology and regulation of 5-lipoxygenase. *American journal of respiratory and critical care medicine* **161**, S11-15

36. Gillmor, S. A., Villasenor, A., Fletterick, R., Sigal, E., and Browner, M. F. (1997) The structure of mammalian 15-lipoxygenase reveals similarity to the lipases and the determinants of substrate specificity. *Nature structural biology* **4**, 1003-1009

37. Hammarberg, T., Provost, P., Persson, B., and Radmark, O. (2000) The N-terminal domain of 5-lipoxygenase binds calcium and mediates calcium stimulation of enzyme activity. *The Journal of biological chemistry* **275**, 38787-38793

38. Pande, A. H., Moe, D., Nemec, K. N., Qin, S., Tan, S., and Tatulian, S. A. (2004) Modulation of human 5-lipoxygenase activity by membrane lipids. *Biochemistry* **43**, 14653-14666

39. Esser, J., Rakonjac, M., Hofmann, B., Fischer, L., Provost, P., Schneider, G., Steinhilber, D., Samuelsson, B., and Radmark, O. (2010) Coactosin-like protein functions as a stabilizing chaperone for 5-lipoxygenase: role of tryptophan 102. *The Biochemical journal* **425**, 265-274

40. Wong, A., Cook, M. N., Foley, J. J., Sarau, H. M., Marshall, P., and Hwang, S. M. (1991) Influx of extracellular calcium is required for the membrane translocation of 5-lipoxygenase and leukotriene synthesis. *Biochemistry* **30**, 9346-9354

41. Kulkarni, S., Das, S., Funk, C. D., Murray, D., and Cho, W. (2002) Molecular basis of the specific subcellular localization of the C2-like domain of 5-lipoxygenase. *The Journal of biological chemistry* **277**, 13167-13174

42. Chen, X. S., and Funk, C. D. (2001) The N-terminal "beta-barrel" domain of 5-lipoxygenase is essential for nuclear membrane translocation. *The Journal of biological chemistry* **276**, 811-818

43. Provost, P., Doucet, J., Hammarberg, T., Gerisch, G., Samuelsson, B., and Radmark, O. (2001) 5-Lipoxygenase interacts with coactosin-like protein. *The Journal of biological chemistry* **276**, 16520-16527

44. Dincbas-Renqvist, V., Pepin, G., Rakonjac, M., Plante, I., Ouellet, D. L., Hermansson, A., Goulet, I., Doucet, J., Samuelsson, B., Radmark, O., and Provost, P. (2009) Human Dicer C-terminus functions as a 5-lipoxygenase binding domain. *Biochimica et biophysica acta* **1789**, 99-108

45. Hammarberg, T., Kuprin, S., Radmark, O., and Holmgren, A. (2001) EPR investigation of the active site of recombinant human 5-lipoxygenase: inhibition by selenide. *Biochemistry* **40**, 6371-6378

46. Minor, W., Steczko, J., Stec, B., Otwinowski, Z., Bolin, J. T., Walter, R., and Axelrod, B. (1996) Crystal structure of soybean lipoxygenase L-1 at 1.4 A resolution. *Biochemistry* **35**, 10687-10701

47. Kobe, M. J., Neau, D. B., Mitchell, C. E., Bartlett, S. G., and Newcomer, M. E. (2014) The structure of human 15-lipoxygenase-2 with a substrate mimic. *The Journal of biological chemistry* **289**, 8562-8569

48. Bundy, G. L., Nidy, E. G., Epps, D. E., Mizsak, S. A., and Wnuk, R. J. (1986) Discovery of an arachidonic acid C-8 lipoxygenase in the gorgonian coral Pseudoplexaura porosa. *The Journal of biological chemistry* **261**, 747-751

49. Schneider, C., Pratt, D. A., Porter, N. A., and Brash, A. R. (2007) Control of oxygenation in lipoxygenase and cyclooxygenase catalysis. *Chemistry & biology* **14**, 473-488

50. Radmark, O., Werz, O., Steinhilber, D., and Samuelsson, B. (2014) 5-Lipoxygenase, a key enzyme for leukotriene biosynthesis in health and disease. *Biochimica et biophysica acta*

51. Percival, M. D., Denis, D., Riendeau, D., and Gresser, M. J. (1992) Investigation of the mechanism of non-turnover-dependent inactivation of purified human 5-lipoxygenase. Inactivation by H2O2 and inhibition by metal ions. *European journal of biochemistry / FEBS* **210**, 109-117

52. Puustinen, T., Scheffer, M. M., and Samuelsson, B. (1988) Regulation of the human leukocyte 5-lipoxygenase: stimulation by micromolar Ca2+ levels and phosphatidylcholine vesicles. *Biochimica et biophysica acta* **960**, 261-267

53. Aharony, D., and Stein, R. L. (1986) Kinetic mechanism of guinea pig neutrophil 5-lipoxygenase. *The Journal of biological chemistry* **261**, 11512-11519

54. Hammarberg, T., and Radmark, O. (1999) 5-lipoxygenase binds calcium. *Biochemistry* **38**, 4441-4447

55. Reddy, K. V., Hammarberg, T., and Radmark, O. (2000) Mg2+ activates 5-lipoxygenase in vitro: dependency on concentrations of phosphatidylcholine and arachidonic acid. *Biochemistry* **39**, 1840-1848

56. Riendeau, D., Falgueyret, J. P., Meisner, D., Sherman, M. M., Laliberte, F., and Street, I. P. (1993) Interfacial catalysis and production of a high ratio of leukotriene A4 to 5-HPETE by 5-lipoxygenase in a coupled assay with phospholipase A2. *Journal of lipid mediators* **6**, 23-30

57. Provost, P., Samuelsson, B., and Radmark, O. (1999) Interaction of 5-lipoxygenase with cellular proteins. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 1881-1885

58. Liepinsh, E., Rakonjac, M., Boissonneault, V., Provost, P., Samuelsson, B., Radmark, O., and Otting, G. (2004) NMR structure of human coactosin-like protein. *Journal of biomolecular NMR* **30**, 353-356

59. Basavarajappa, D., Wan, M., Lukic, A., Steinhilber, D., Samuelsson, B., and Radmark, O. (2014) Roles of coactosin-like protein (CLP) and 5-lipoxygenase-activating protein (FLAP) in cellular leukotriene biosynthesis. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 11371-11376

60. Ochi, K., Yoshimoto, T., Yamamoto, S., Taniguchi, K., and Miyamoto, T. (1983) Arachidonate 5-lipoxygenase of guinea pig peritoneal polymorphonuclear leukocytes. Activation by adenosine 5'-triphosphate. *The Journal of biological chemistry* **258**, 5754-5758

61. Werz, O., Burkert, E., Samuelsson, B., Radmark, O., and Steinhilber, D. (2002) Activation of 5-lipoxygenase by cell stress is calcium independent in human polymorphonuclear leukocytes. *Blood* **99**, 1044-1052

62. Zhang, Y. Y., Hammarberg, T., Radmark, O., Samuelsson, B., Ng, C. F., Funk, C. D., and Loscalzo, J. (2000) Analysis of a nucleotide-binding site of 5-lipoxygenase by affinity labelling: binding characteristics and amino acid sequences. *The Biochemical journal* **351 Pt 3**, 697-707

63. Hafner, A. K., Cernescu, M., Hofmann, B., Ermisch, M., Hornig, M., Metzner, J., Schneider, G., Brutschy, B., and Steinhilber, D. (2011) Dimerization of human 5-lipoxygenase. *Biological chemistry* **392**, 1097-1111

64. Okamoto, H., Hammarberg, T., Zhang, Y. Y., Persson, B., Watanabe, T., Samuelsson, B., and Radmark, O. (2005) Mutation analysis of the human 5-lipoxygenase C-terminus: support for a stabilizing C-terminal loop. *Biochimica et biophysica acta* **1749**, 123-131

65. Walther, M., Hofheinz, K., Vogel, R., Roffeis, J., and Kuhn, H. (2011) The N-terminal beta-barrel domain of mammalian lipoxygenases including mouse 5-lipoxygenase is not essential for catalytic activity and membrane binding but exhibits regulatory functions. *Archives of biochemistry and biophysics* **516**, 1-9

66. Pergola, C., Gaboriaud-Kolar, N., Jestadt, N., Konig, S., Kritsanida, M., Schaible, A. M., Li, H., Garscha, U., Weinigel, C., Barz, D., Albring, K. F., Huber, O., Skaltsounis, A. L., and Werz, O. (2014) Indirubin core structure of glycogen synthase kinase-3 inhibitors as novel chemotype for intervention with 5-lipoxygenase. *Journal of medicinal chemistry* **57**, 3715-3723

67. Smith, W. L., and Lands, W. E. (1972) Oxygenation of polyunsaturated fatty acids during prostaglandin biosynthesis by sheep vesicular gland. *Biochemistry* **11**, 3276-3285

68. Riendeau, D., Denis, D., Choo, L. Y., and Nathaniel, D. J. (1989) Stimulation of 5-lipoxygenase activity under conditions which promote lipid peroxidation. *The Biochemical journal* **263**, 565-572

69. Radmark, O. (2002) Arachidonate 5-lipoxygenase. *Prostaglandins & other lipid mediators* **68-69**, 211-234

70. Werz, O., and Steinhilber, D. (2006) Therapeutic options for 5-lipoxygenase inhibitors. *Pharmacology & therapeutics* **112**, 701-718

71. Werz, O., Klemm, J., Samuelsson, B., and Radmark, O. (2001) Phorbol ester up-regulates capacities for nuclear translocation and phosphorylation of 5-lipoxygenase in Mono Mac 6 cells and human polymorphonuclear leukocytes. *Blood* **97**, 2487-2495

72. Werz, O., Szellas, D., Steinhilber, D., and Radmark, O. (2002) Arachidonic acid promotes phosphorylation of 5-lipoxygenase at Ser-271 by MAPK-activated protein kinase 2 (MK2). *The Journal of biological chemistry* **277**, 14793-14800

73. Lepley, R. A., and Fitzpatrick, F. A. (1996) Inhibition of mitogen-activated protein kinase kinase blocks activation and redistribution of 5-lipoxygenase in HL-60 cells. *Archives of biochemistry and biophysics* **331**, 141-144

74. Werz, O., Klemm, J., Samuelsson, B., and Radmark, O. (2000) 5-lipoxygenase is phosphorylated by p38 kinase-dependent MAPKAP kinases. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 5261-5266

75. Werz, O., Burkert, E., Fischer, L., Szellas, D., Dishart, D., Samuelsson, B., Radmark, O., and Steinhilber, D. (2003) 5-Lipoxygenase activation by MAPKAPK-2 and ERKs. *Advances in experimental medicine and biology* **525**, 129-132

76. Fredman, G., Ozcan, L., Spolitu, S., Hellmann, J., Spite, M., Backs, J., and Tabas, I. (2014) Resolvin D1 limits 5-lipoxygenase nuclear localization and leukotriene B4 synthesis by inhibiting a calcium-activated kinase pathway. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 14530-14535

77. Luo, M., Jones, S. M., Phare, S. M., Coffey, M. J., Peters-Golden, M., and Brock, T. G. (2004) Protein kinase A inhibits leukotriene synthesis by phosphorylation of 5-lipoxygenase on serine 523. *The Journal of biological chemistry* **279**, 41512-41520

78. Flamand, N., Surette, M. E., Picard, S., Bourgoin, S., and Borgeat, P. (2002) Cyclic AMP-mediated inhibition of 5-lipoxygenase translocation and leukotriene biosynthesis in human neutrophils. *Molecular pharmacology* **62**, 250-256

79. Flamand, N., Lefebvre, J., Surette, M. E., Picard, S., and Borgeat, P. (2006) Arachidonic acid regulates the translocation of 5-lipoxygenase to the nuclear membranes in human neutrophils. *The Journal of biological chemistry* **281**, 129-136

80. Luo, M., Jones, S. M., Peters-Golden, M., and Brock, T. G. (2003) Nuclear localization of 5-lipoxygenase as a determinant of leukotriene B4 synthetic capacity. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 12165-12170

81. Bouman, A., Heineman, M. J., and Faas, M. M. (2005) Sex hormones and the immune response in humans. *Human reproduction update* **11**, 411-423

82. Pergola, C., Dodt, G., Rossi, A., Neunhoeffer, E., Lawrenz, B., Northoff, H., Samuelsson, B., Radmark, O., Sautebin, L., and Werz, O. (2008) ERK-mediated regulation of leukotriene biosynthesis by androgens: a molecular basis for gender differences in inflammation and asthma. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 19881-19886

83. Pergola, C., Rogge, A., Dodt, G., Northoff, H., Weinigel, C., Barz, D., Radmark, O., Sautebin, L., and Werz, O. (2011) Testosterone suppresses phospholipase D, causing sex differences in leukotriene biosynthesis in human monocytes. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **25**, 3377-3387

84. Rossi, A., Pergola, C., Pace, S., Radmark, O., Werz, O., and Sautebin, L. (2014) In vivo sex differences in leukotriene biosynthesis in zymosan-induced peritonitis. *Pharmacological research : the official journal of the Italian Pharmacological Society* **87**, 1-7

85. Goulet, J. L., Griffiths, R. C., Ruiz, P., Spurney, R. F., Pisetsky, D. S., Koller, B. H., and Coffman, T. M. (1999) Deficiency of 5-lipoxygenase abolishes sex-related survival differences in MRL-lpr/lpr mice. *Journal of immunology* **163**, 359-366

86. Ueda, N., Kaneko, S., Yoshimoto, T., and Yamamoto, S. (1986) Purification of arachidonate 5-lipoxygenase from porcine leukocytes and its reactivity with hydroperoxyeicosatetraenoic acids. *The Journal of biological chemistry* **261**, 7982-7988

87. Ford-Hutchinson, A. W., Gresser, M., and Young, R. N. (1994) 5-Lipoxygenase. *Annual review of biochemistry* **63**, 383-417

88. De Carolis, E., Denis, D., and Riendeau, D. (1996) Oxidative inactivation of human 5-lipoxygenase in phosphatidylcholine vesicles. *European journal of biochemistry / FEBS* **235**, 416-423

89. Aharony, D., Redkar-Brown, D. G., Hubbs, S. J., and Stein, R. L. (1987) Kinetic studies on the inactivation of 5-lipoxygenase by 5(S)-hydroperoxyeicosatetraenoic acid. *Prostaglandins* **33**, 85-100

90. Lepley, R. A., and Fitzpatrick, F. A. (1994) Irreversible inactivation of 5-lipoxygenase by leukotriene A4. Characterization of product inactivation with purified enzyme and intact leukocytes. *The Journal of biological chemistry* **269**, 2627-2631

91. Gillard, J., Ford-Hutchinson, A. W., Chan, C., Charleson, S., Denis, D., Foster, A., Fortin, R., Leger, S., McFarlane, C. S., Morton, H., and et al. (1989) L-663,536 (MK-886) (3-[1-(4-chlorobenzyl)-3-t-butyl-thio-5-isopropylindol-2-yl]-2,2 - dimethylpropanoic acid), a novel, orally active leukotriene biosynthesis inhibitor. *Canadian journal of physiology and pharmacology* **67**, 456-464

92. Muller-Peddinghaus, R., Fruchtmann, R., Ahr, H. J., Beckermann, B., Buhner, K., Fugmann, B., Junge, B., Matzke, M., Kohlsdorfer, C., Raddatz, S., and et al. (1993) BAY X1005, a new selective inhibitor of leukotriene synthesis: pharmacology and pharmacokinetics. *Journal of lipid mediators* **6**, 245-248

93. Brideau, C., Chan, C., Charleson, S., Denis, D., Evans, J. F., Ford-Hutchinson, A. W., Fortin, R., Gillard, J. W., Guay, J., Guevremont, D., and et al. (1992) Pharmacology of MK-0591 (3-[1-(4-chlorobenzyl)-3-(t-butylthio)-5-(quinolin-2-yl-methoxy)- indol-2-yl]-2,2-dimethyl propanoic acid), a potent, orally active leukotriene biosynthesis inhibitor. *Canadian journal of physiology and pharmacology* **70**, 799-807

94. Vickers, P. J., Adam, M., Charleson, S., Coppolino, M. G., Evans, J. F., and Mancini, J. A. (1992) Identification of amino acid residues of 5-lipoxygenase-activating protein essential for the binding of leukotriene biosynthesis inhibitors. *Molecular pharmacology* **42**, 94-102

95. Charleson, S., Prasit, P., Leger, S., Gillard, J. W., Vickers, P. J., Mancini, J. A., Charleson, P., Guay, J., Ford-Hutchinson, A. W., and Evans, J. F. (1992) Characterization of a 5-lipoxygenase-activating protein binding assay: correlation of affinity for 5-lipoxygenase-activating protein with leukotriene synthesis inhibition. *Molecular pharmacology* **41**, 873-879

96. Friedman, B. S., Bel, E. H., Buntinx, A., Tanaka, W., Han, Y. H., Shingo, S., Spector, R., and Sterk, P. (1993) Oral leukotriene inhibitor (MK-886) blocks allergen-induced airway responses. *The American review of respiratory disease* **147**, 839-844

97. Dahlen, B., Kumlin, M., Ihre, E., Zetterstrom, O., and Dahlen, S. E. (1997) Inhibition of allergen-induced airway obstruction and leukotriene generation in atopic asthmatic subjects by the leukotriene biosynthesis inhibitor BAYx 1005. *Thorax* **52**, 342-347

98. Diamant, Z., Timmers, M. C., van der Veen, H., Friedman, B. S., De Smet, M., Depre, M., Hilliard, D., Bel, E. H., and Sterk, P. J. (1995) The effect of MK-0591, a novel 5-lipoxygenase activating protein inhibitor, on leukotriene biosynthesis and allergen-induced airway responses in asthmatic subjects in vivo. *The Journal of allergy and clinical immunology* **95**, 42-51

99. Kent, S. E., Bentley, J. H., Miller, D., Sterling, R., Menendez, R., Tarpay, M., Pearlman, D. S., and Norris, V. (2014) The effect of GSK2190915, a 5-lipoxygenase-activating protein inhibitor, on exercise-induced bronchoconstriction. *Allergy and asthma proceedings : the official journal of regional and state allergy societies* **35**, 126-133

100. Ferguson, A. D., McKeever, B. M., Xu, S., Wisniewski, D., Miller, D. K., Yamin, T. T., Spencer, R. H., Chu, L., Ujjainwalla, F., Cunningham, B. R., Evans, J. F., and Becker, J. W. (2007) Crystal structure of inhibitor-bound human 5-lipoxygenase-activating protein. *Science* **317**, 510-512

101. Poeckel, D., and Werz, O. (2006) Boswellic acids: biological actions and molecular targets. *Current medicinal chemistry* **13**, 3359-3369

102. Poeckel, D., Tausch, L., Kather, N., Jauch, J., and Werz, O. (2006) Boswellic acids stimulate arachidonic acid release and 12-lipoxygenase activity in human platelets independent of Ca2+ and differentially interact with platelet-type 12-lipoxygenase. *Molecular pharmacology* **70**, 1071-1078

103. Brash, A. R. (2001) Arachidonic acid as a bioactive molecule. *The Journal of clinical investigation* **107**, 1339-1345

104. Hakonarson, H. (2006) Role of FLAP and PDE4D in myocardial infarction and stroke: target discovery and future treatment options. *Current treatment options in cardiovascular medicine* **8**, 183-192

105. Jakobsson, P. J., Morgenstern, R., Mancini, J., Ford-Hutchinson, A., and Persson, B. (1999) Common structural features of MAPEG -- a widespread superfamily of membrane associated proteins with highly divergent functions in eicosanoid and glutathione metabolism. *Protein science : a publication of the Protein Society* **8**, 689-692

106. Mandal, A. K., Jones, P. B., Bair, A. M., Christmas, P., Miller, D., Yamin, T. T., Wisniewski, D., Menke, J., Evans, J. F., Hyman, B. T., Bacskai, B., Chen, M., Lee, D. M., Nikolic, B., and Soberman, R. J. (2008) The nuclear membrane organization of leukotriene synthesis. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 20434-20439

107. Mancini, J. A., Abramovitz, M., Cox, M. E., Wong, E., Charleson, S., Perrier, H., Wang, Z., Prasit, P., and Vickers, P. J. (1993) 5-lipoxygenase-activating protein is an arachidonate binding protein. *FEBS letters* **318**, 277-281

108. Byrum, R. S., Goulet, J. L., Griffiths, R. J., and Koller, B. H. (1997) Role of the 5-lipoxygenase-activating protein (FLAP) in murine acute inflammatory responses. *The Journal of experimental medicine* **185**, 1065-1075

109. Rouzer, C. A., Rands, E., Kargman, S., Jones, R. E., Register, R. B., and Dixon, R. A. (1988) Characterization of cloned human leukocyte 5-lipoxygenase expressed in mammalian cells. *The Journal of biological chemistry* **263**, 10135-10140

110. Rouzer, C. A., Ford-Hutchinson, A. W., Morton, H. E., and Gillard, J. W. (1990) MK886, a potent and specific leukotriene biosynthesis inhibitor blocks and reverses the membrane association of 5-lipoxygenase in ionophore-challenged leukocytes. *The Journal of biological chemistry* **265**, 1436-1442

111. Strid, T., Svartz, J., Franck, N., Hallin, E., Ingelsson, B., Soderstrom, M., and Hammarstrom, S. (2009) Distinct parts of leukotriene C(4) synthase interact with 5-lipoxygenase and 5-lipoxygenase activating protein. *Biochemical and biophysical research communications* **381**, 518-522

112. Zarini, S., Gijon, M. A., Folco, G., and Murphy, R. C. (2006) Effect of arachidonic acid reacylation on leukotriene biosynthesis in human neutrophils stimulated with granulocyte-macrophage colony-stimulating factor and formyl-methionyl-leucyl-phenylalanine. *The Journal of biological chemistry* **281**, 10134-10142

113. Clark, J. D., Lin, L. L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N., and Knopf, J. L. (1991) A novel arachidonic acid-selective cytosolic PLA2 contains a Ca(2+)-dependent translocation domain with homology to PKC and GAP. *Cell* **65**, 1043-1051

114. Schievella, A. R., Regier, M. K., Smith, W. L., and Lin, L. L. (1995) Calcium-mediated translocation of cytosolic phospholipase A2 to the nuclear envelope and endoplasmic reticulum. *The Journal of biological chemistry* **270**, 30749-30754

115. Dessen, A., Tang, J., Schmidt, H., Stahl, M., Clark, J. D., Seehra, J., and Somers, W. S. (1999) Crystal structure of human cytosolic phospholipase A2 reveals a novel topology and catalytic mechanism. *Cell* **97**, 349-360

116. Lin, L. L., Wartmann, M., Lin, A. Y., Knopf, J. L., Seth, A., and Davis, R. J. (1993) cPLA2 is phosphorylated and activated by MAP kinase. *Cell* **72**, 269-278

117. Uozumi, N., Kume, K., Nagase, T., Nakatani, N., Ishii, S., Tashiro, F., Komagata, Y., Maki, K., Ikuta, K., Ouchi, Y., Miyazaki, J., and Shimizu, T. (1997) Role of cytosolic phospholipase A2 in allergic response and parturition. *Nature* **390**, 618-622

118. Surette, M. E., and Chilton, F. H. (1998) The distribution and metabolism of arachidonate-containing phospholipids in cellular nuclei. *The Biochemical journal* **330 (Pt 2)**, 915-921

119. Luo, M., Jones, S. M., Flamand, N., Aronoff, D. M., Peters-Golden, M., and Brock, T. G. (2005) Phosphorylation by protein kinase a inhibits nuclear import of 5-lipoxygenase. *The Journal of biological chemistry* **280**, 40609-40616

120. Bair, A. M., Turman, M. V., Vaine, C. A., Panettieri, R. A., Jr., and Soberman, R. J. (2012) The nuclear membrane leukotriene synthetic complex is a signal integrator and transducer. *Molecular biology of the cell* **23**, 4456-4464

121. Rouzer, C. A., and Kargman, S. (1988) Translocation of 5-lipoxygenase to the membrane in human leukocytes challenged with ionophore A23187. *The Journal of biological chemistry* **263**, 10980-10988

122. Kargman, S., Vickers, P. J., and Evans, J. F. (1992) A23187-induced translocation of 5-lipoxygenase in osteosarcoma cells. *The Journal of cell biology* **119**, 1701-1709

123. Sala, A., Folco, G., and Murphy, R. C. (2010) Transcellular biosynthesis of eicosanoids. *Pharmacological reports : PR* **62**, 503-510

124. Niegowski, D., Kleinschmidt, T., Olsson, U., Ahmad, S., Rinaldo-Matthis, A., and Haeggstrom, J. Z. (2014) Crystal structures of leukotriene C4 synthase in complex with product analogs: implications for the enzyme mechanism. *The Journal of biological chemistry* **289**, 5199-5207

125. Martinez Molina, D., Eshaghi, S., and Nordlund, P. (2008) Catalysis within the lipid bilayer-structure and mechanism of the MAPEG family of integral membrane proteins. *Current opinion in structural biology* **18**, 442-449

126. Lam, B. K., and Austen, K. F. (2002) Leukotriene C4 synthase: a pivotal enzyme in cellular biosynthesis of the cysteinyl leukotrienes. *Prostaglandins & other lipid mediators* **68-69**, 511-520

127. Bisgaard, H. (2001) Pathophysiology of the cysteinyl leukotrienes and effects of leukotriene receptor antagonists in asthma. *Allergy* **56 Suppl 66**, 7-11

128. Soderstrom, M., Mannervik, B., Garkov, V., and Hammarstrom, S. (1992) On the nature of leukotriene C4 synthase in human platelets. *Archives of biochemistry and biophysics* **294**, 70-74

129. Thunnissen, M. M., Nordlund, P., and Haeggstrom, J. Z. (2001) Crystal structure of human leukotriene A(4) hydrolase, a bifunctional enzyme in inflammation. *Nature structural biology* **8**, 131-135

130. Haeggstrom, J. Z., and Wetterholm, A. (2002) Enzymes and receptors in the leukotriene cascade. *Cellular and molecular life sciences : CMLS* **59**, 742-753

131. Stsiapanava, A., Olsson, U., Wan, M., Kleinschmidt, T., Rutishauser, D., Zubarev, R. A., Samuelsson, B., Rinaldo-Matthis, A., and Haeggstrom, J. Z. (2014) Binding of Pro-Gly-Pro at the active site of leukotriene A4 hydrolase/aminopeptidase and development of an epoxide hydrolase selective inhibitor. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 4227-4232

132. Snelgrove, R. J., Jackson, P. L., Hardison, M. T., Noerager, B. D., Kinloch, A., Gaggar, A., Shastry, S., Rowe, S. M., Shim, Y. M., Hussell, T., and Blalock, J. E. (2010) A critical role for LTA4H in limiting chronic pulmonary neutrophilic inflammation. *Science* **330**, 90-94

133. Serhan, C. N. (1994) Lipoxin biosynthesis and its impact in inflammatory and vascular events. *Biochimica et biophysica acta* **1212**, 1-25

134. Singh, D., Boyce, M., Norris, V., Kent, S. E., and Bentley, J. H. (2013) Inhibition of the early asthmatic response to inhaled allergen by the 5-lipoxygenase activating protein inhibitor GSK2190915: a dose-response study. *International journal of general medicine* **6**, 897-903

135. Evans, J. F., Leville, C., Mancini, J. A., Prasit, P., Therien, M., Zamboni, R., Gauthier, J. Y., Fortin, R., Charleson, P., MacIntyre, D. E., and et al. (1991) 5-Lipoxygenase-activating protein is the target of a quinoline class of leukotriene synthesis inhibitors. *Molecular pharmacology* **40**, 22-27

136. Chen, X. S., Zhang, Y. Y., and Funk, C. D. (1998) Determinants of 5-lipoxygenase nuclear localization using green fluorescent protein/5-lipoxygenase fusion proteins. *The Journal of biological chemistry* **273**, 31237-31244

137. Werz, O., and Steinhilber, D. (2005) Development of 5-lipoxygenase inhibitors--lessons from cellular enzyme regulation. *Biochemical pharmacology* **70**, 327-333

138. Fischer, L., Hornig, M., Pergola, C., Meindl, N., Franke, L., Tanrikulu, Y., Dodt, G., Schneider, G., Steinhilber, D., and Werz, O. (2007) The molecular mechanism of the inhibition by licofelone of the biosynthesis of 5-lipoxygenase products. *British journal of pharmacology* **152**, 471-480

139. Woods, J. W., Evans, J. F., Ethier, D., Scott, S., Vickers, P. J., Hearn, L., Heibein, J. A., Charleson, S., and Singer, II. (1993) 5-lipoxygenase and 5-lipoxygenase-activating protein are localized in the nuclear envelope of activated human leukocytes. *The Journal of experimental medicine* **178**, 1935-1946

140. Surette, M. E., Dallaire, N., Jean, N., Picard, S., and Borgeat, P. (1998) Mechanisms of the priming effect of lipopolysaccharides on the biosynthesis of leukotriene B4 in chemotactic peptide-stimulated human neutrophils. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **12**, 1521-1531

141. Schaible, A. M., Koeberle, A., Northoff, H., Lawrenz, B., Weinigel, C., Barz, D., Werz, O., and Pergola, C. (2013) High capacity for leukotriene biosynthesis in peripheral blood during pregnancy. *Prostaglandins, leukotrienes, and essential fatty acids* **89**, 245-255

142. Carter, G. W., Young, P. R., Albert, D. H., Bouska, J., Dyer, R., Bell, R. L., Summers, J. B., and Brooks, D. W. (1991) 5-lipoxygenase inhibitory activity of zileuton. *The Journal of pharmacology and experimental therapeutics* **256**, 929-937

APPENDIX 1: ACKNOWLEDGEMENTS

I would like to thank Prof. Dr. Oliver Werz for being an excellent supervisor and mentor to me. Prof. Werz introduced me into the field of lipid-mediated inflammation, and draw my interests to the 5-LO pathway, and molecular biology. He has tought me to become a successful researcher, and has opened every possible door for me to embrace my career.

Special thanks to Prof. Dr. Gerhard Scriba for reviewing my dissertation, and for the kind help during my teaching time at the university, especially regarding discussions to „Quanatitative Analyse von Arzneistoffen“.

I would like to thank Prof. Dr. Sabine Grösch from the University of Frankfurt for the kind agreement to review my dissertation.

Next, I would like to thank my advisor and mentor Dr. Ulrike Garscha for being a brilliant and outstanding researcher to work with. Ulrike has helped and encouraged me in every possible way during my time as a Ph.D student. Working with Ulrike was attended by fruitful discussions, innovative ideas, the experience to learn something new every day, and to be critical about myself and my data. She always supported my enthusiasm and curiosity for molecular biology and the 5-LO pathway. Finally, Ulrike is much more than simply my supervisor – in fact she became a friend!

I also would like to thank Prof. Dr. Marcia Newcomer for being a mentor and valuable friend at the same time for me. Marcia has given me the opportunity to work in her laboratory at Louisiana State University (USA) for eight weeks, and introduced me into the field of structural biology. Talking to Marcia always encouraged me to look deeper at my data and never give up.

I thank Prof. Dr. O. Rådmark for fruitful discussions regarding the 5-LO/CLP interaction and 5-LO antibody supply.

I also want to thank my collaberation partners for the effient und highly productive work together. Especially, Prof. Dr. Erden Banoglu and his group in Ankara for the synthesis of the FLAP inhibitor BRP-7 (and derivatives). Prof. Dr. Dieter Steinhilber for plasmids and 5-LO antibody supply as well as interesting discussions reagarding the 5-LO/FLAP interaction, and all members of the Institute for transfusion medicine at the hospital in Jena, especially Dr. C. Weinigel and Dr. S. Rummler, for buffy coat supply.

I thank Dr. Andreas Koeberle for introducing me into the field of UPLC-MS/MS and mPGES-1 inhibition.

I would like to thank the one person without whom the daily life in the lab would not have been so easy, funny, critical and productive at the same time – Bettina Mönch. We always supported and believed in each other, one of many reasons why we became so good friends.

Next, I would like to thank Erik Romp, who became a valuable friend to me that is always there for me in research and private matters. You have been a great diploma student to supervise and work with.

Special thanks to my two favourite italian Ph.D. Simona Pace, not only for doing the animal experiments for me. Thank you for your support and friendship.

Finally, I would like to thank all the members of the Prof. Werz and Prof. Scriba group for being kind, helpful, and supportive every day. Everybody did his best to create a fantastic and efficient working atmosphere. Especially, Katrin Fischer who always kept the overview about chemical orders, HPLCs, and LC-MS/MS samples with a smile on her face.

WIDMUNG

Großer Dank gebührt meinen Eltern, Annette und Otmar Gerstmeier, die immer für mich da sind und mich stets in meiner akademischen Laufbahn unterstützt haben, wohlwissend wie wichtig Bildung für die persönliche Entwicklung ist. Ich danke meinen beiden „kleinen“ Brüdern: Fabian und Adrian, sowie meinen Großeltern: Ursel & Helmut und Brigitta & Werner, die mich jeden Tag daran erinnern, dass Familie unser wichtigstes Gut im Leben ist. Weiterhin danke ich aufrichtig Frank and Kerstin Giesel für ihre stetige Unterstützung, Motivation und herzliche Aufnahme in ihre Familie.

Außerdem widme ich diese Arbeit meiner Freundschaft zu fünf fantastischen starken Frauen, die ich seit der Grundschule kenne: Heidi, Kristin, Corinna, Mirjam und Theresa – Mein Leben wäre nicht dasselbe ohne euch!

Mein größter Dank gilt meinem Partner, Carsten Giesel. Dich zu treffen, veränderte mein ganzes Leben. Du machst mich zu einem besseren Menschen und zeigst mir jeden Tag, dass es sich lohnt für seine Träume und Ziele zu kämpfen, weil du an mich glaubst! Nichts von all dem wäre möglich gewesen ohne deine Stärke, Vertrauen und endlose Liebe zu mir.

APPENDIX 2*:* CURRICULUM VITAE

PERSON

Name: **Jana Karin Gerstmeier**

Geburtstag: 15. Januar 1984, Mühlhausen; Thüringen

Nationalität: deutsch

Familienstand: ledig

AUSBILDUNG

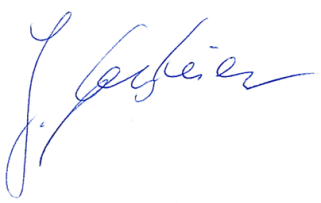
Sept. 1994 – Juni 2002	Käthe-Kollwitz-Gymnasium, Lengenfeld unterm Stein Allgemeine Hochschulreife (Abitur)
Okt. 2005 – Sept. 2009	Studium der Pharmazie an der Friedrich-Schiller-Universität Jena; Zweites Staatsexamen (Note: 1,6)
Okt. 2009 – April 2010	Diplomarbeit am Institut für zelluläre und molekulare Immunologie an der Georg-August-Universität Göttingen (Prof. Dr. Jürgen Wienands); Dipl.-Pharm. (Mai 2011; Note: 1,0)
Mai 2010 – Okt. 2010	Pharmaziepraktikant, Turm-Apotheke; Duderstadt
Dez. 2010	Drittes Staatsexamen Pharmazie (Note: 1,0) Approbation als Apotheker
seit März 2011	Wissenschaftlicher Mitarbeiter am Lehrstuhl für Pharmazeutische/Medizinische Chemie an der Friedrich-Schiller-Universität Jena (Prof. Dr. Oliver Werz)

WISSENSCHAFTLICHE TÄTIGKEITEN UND AUFENTHALTE

März 2007 – Sept. 2009	Stipendium für das Pharmaziestudium durch das Reemtsma Begabtenförderungswerk, Hamburg
Feb 2013 – April 2013	Forschungsaufenthalt am Department of Biological Sciences an der Louisiana State University; USA (Prof. Dr. Marcia Newcomer)

AKTIVE TEILNAHME AN KONGRESSEN UND WISSENSCHAFTLICHEN MEETINGS

August 27 - 31, 2012:	„Frankfurt International Research School for Translational Biomedicine (FIRST) Summer School“; Löwenstein, Deutschland **(Vortrag)**
Sept. 27 - 28, 2012:	“4th European Workshop on Lipid Mediators”; Paris, Frankreich **(Poster)**
Januar 22 - 23, 2013:	“8th Status Seminar Chemical Biology"; Frankfurt, Deutschland **(Poster)**
Oktober 22, 2014	“Educational Session 5th European Workshop on Lipid Mediators“; Istanbul, Türkei
Oktober 23 - 24, 2014	“5th European Workshop on Lipid Mediators“; Istanbul, Türkei **(Poster)**
July 12 – 15, 2015	14th International Conference on Bioactive Lipids in Cancer, Inflammation and Related Diseases **(Poster)**



Jena, den 25.06.2015 Jana Gerstmeier

APPENDIX 3: LIST OF PUBLICATIONS

11. Gerstmeier, J., ME. Newcomer, S. Dennhardt, O. Werz and U. Garscha (2015). "The regulatory role of FLAP in 5-LOX substrate acquisition and turnover." **J Biol Chem.**, in preparation.

10. Gerstmeier, J., C. Weinigel, S.Rummler, O. Werz and U. Garscha (2015). "Time-resolved in situ assembly of the leukotriene synthetic 5-lipoxygenase/5-lipoxygenase-activating protein (FLAP) complex in primary leukocytes." **FASEB J**, under review after invitation to submit

9. Çelikoğlu, E., S. Völker, A. Olgaç, J. Gerstmeier, U. Garscha, B. Çalışkan, A. Carotti, S. Levent, A. Macchiarulo, O. Werz, E Banoglu (2015). „4,5-Diarylisoxazole-3-carboxylic acids: a new class of leukotriene biosynthesis inhibitors dually acting on 5-lipoxygenase (5-LO) and 5-lipoxygenase activating protein (FLAP)." **J Med Chem.**, submitted.

8. Häfner, H.A., J. Gerstmeier, M. Hörnig, S. George, A.K. Ball, M. Schröder, U. Garscha, O. Werz and D. Steinhilber (2015). "Characterization of the interaction of human 5-lipoxygenase with its activating protein FLAP." **Biochim Biophys Acta**, in revision.

7. Gerstmeier, J., C. Weinigel, D. Barz, O. Werz and U. Garscha (2014). "An experimental cell-based model for studying the cell biology and molecular pharmacology of 5-lipoxygenase-activating protein in leukotriene biosynthesis." **Biochim Biophys Acta** 1840(9): 2961-2969.

6. Pergola, C., J. Gerstmeier, B. Mönch, B. Caliskan, S. Luderer, C. Weinigel, D. Barz, J. Maczewsky, S. Pace, A. Rossi, L. Sautebin, E. Banoglu and O. Werz (2014). "The novel benzimidazole derivative BRP-7 inhibits leukotriene biosynthesis in vitro and in vivo by targeting 5-lipoxygenase-activating protein (FLAP)." **Br J Pharmacol** 171(12): 3051-3064.

5. Napagoda, M., J. Gerstmeier, A. Koeberle, S. Wesely, S. Popella, S. Lorenz, K. Scheubert, S. Bocker, A. Svatos and O. Werz (2014). "Munronia pinnata (Wall.) Theob.: unveiling phytochemistry and dual inhibition of 5-lipoxygenase and microsomal prostaglandin E2 synthase (mPGES)-1." **J Ethnopharmacol** 151(2): 882-890.

4. Napagoda, M., J. Gerstmeier, S. Wesely, S. Popella, S. Lorenz, K. Scheubert, A. Svatos and O. Werz (2014). "Inhibition of 5-lipoxygenase as anti-inflammatory mode of action of Plectranthus zeylanicus Benth and chemical characterization of ingredients by a mass spectrometric approach." **J Ethnopharmacol** 151(2): 800-809.

3. Sardella, R., S. Levent, F. Ianni, B. Caliskan, J. Gerstmeier, C. Pergola, O. Werz, E. Banoglu and B. Natalini (2014). "Chromatographic separation and biological evaluation of benzimidazole derivative enantiomers as inhibitors of leukotriene biosynthesis." **J Pharm Biomed Anal** 89: 88-92.

2. Oettl, S. K., J. Gerstmeier, S. Y. Khan, K. Wiechmann, J. Bauer, A. G. Atanasov, C. Malainer, E. M. Awad, P. Uhrin, E. H. Heiss, B. Waltenberger, D. Remias, J. M. Breuss, J. Boustie, V. M. Dirsch, H. Stuppner, O. Werz and J. M. Rollinger (2013). "Imbricaric acid and perlatolic acid: multi-targeting anti-inflammatory depsides from Cetrelia monachorum." **PLOS One** 8(10): e76929.

1. Banoglu, E., B. Caliskan, S. Luderer, G. Eren, Y. Ozkan, W. Altenhofen, C. Weinigel, D. Barz, J. Gerstmeier, C. Pergola and O. Werz (2012). "Identification of novel benzimidazole derivatives as inhibitors of leukotriene biosynthesis by virtual screening targeting 5-lipoxygenase-activating protein (FLAP)." **Bioorg Med Chem** 20(12): 3728-3741.

APPENDIX 4: AUTHOR CONTRIBUTION STATEMENT

Manuscript I

Pergola, C., J. Gerstmeier, B. Mönch, B. Caliskan, S. Luderer, C. Weinigel, D. Barz, J. Maczewsky, S. Pace, A. Rossi, L. Sautebin, E. Banoglu and O. Werz (2014). "The novel benzimidazole derivative BRP-7 inhibits leukotriene biosynthesis *in vitro* and *in vivo* by targeting 5-lipoxygenase-activating protein (FLAP)." **Br J Pharmacol** 171(12): 3051-3064.

author	**contribution**
J. Gerstmeier	cell culture and performance of blood cell isolation, purification of recombinant human 5-LO from E.coli, analysis of [^{3}H]-AA release, evaluation of BRP-7 derivatives as FLAP inhibitors, HPLC analysis of 5-LO products, mPGS-1 assay, immunofluorescence microscopy of 5-LO in monocytes, analysis of data and preparation of graphs, analysis of statistics, co-writing of the manuscript **total contribution: 50%**
C.Pergola	experimental design, whole blood assay, immunofluorescence microscopy of neutrophils, writing the manuscript
B.Mönch	SDS-PAGE and Western Blot
B. Caliskan, E. Banoglu	chemical synthesis and supply of BRP-7
S. Luderer	inhibitor studies in isolated human monocytes and neutrophils
C. Weinigel, D. Barz	blood withdrawn and preparation of human leukocyte concentrates (buffy coats)
J. Maczewsky, S. Pace, A. Rossi, L. Sautebin	*in vivo* experiments
O. Werz	experimental design, conceiving the project, writing the manuscript

Manuscript II

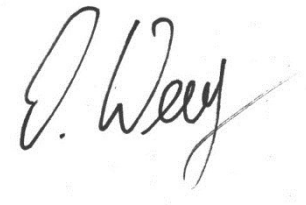
Gerstmeier, J., C. Weinigel, D. Barz, O. Werz and U. Garscha (2014). "An experimental cell-based model for studying the cell biology and molecular pharmacology of 5-lipoxygenase-activating protein in leukotriene biosynthesis." **Biochim. Biophys. Acta** 1840(9): 2961-2969.

author	**contribution**
J. Gerstmeier	cloning of FLAP sequence into mammalian expression plasmid, stable transfection of HEK293 cells with pcDNA3.1/neom(+)_5-LO and pcDNA3.1/hygro(-)_FLAP, purification of human leukocytes, 5-LO activity assays and inhibitor studies in HEK293 cells, monocytes, and neutrophils, HPLC analysis of 5-LO products, immunofluorescence microscopy, SDS-page and Western blot, [^{3}H]-AA release, analysis of data and preparation of graphs, analysis of statistics, writing the manuscript **total contribution: 80%**
C. Weinigel, D. Barz	blood withdrawn and preparation of human leukocyte concentrates (buffy coats)
O. Werz	design of study, writing the manuscript
U. Garscha	discussion and supervising experiments, writing the manuscript

Manuscript III

Gerstmeier, J., C. Weinigel, S. Rummler, O. Rådmark, O. Werz and U. Garscha (2015). "Time-resolved in situ assembly of the leukotriene-synthetic 5-LO / FLAP complex in blood leukocytes." **FASEB J**, under review after invitation to submit.

author	**contribution**
J. Gerstmeier	purification of human monocytes and neutrophils, 5-LO activity assay in intact cells and cell homogenates, HPLC analysis of 5-LO products, stable transfection of HEK293 cells, immunofluorescence microscopy, proximity ligation assay, [^{3}H]-AA release, SDS-page and Western blot, data analysis and preparation of graphs, analysis of statistics, writing the manuscript **total contribution: 80%**
C. Weinigel, S.Rummler	blood withdrawn and preparation of human leukocyte concentrates (buffy coats)
O. Rådmark	discussions regarding CLP/5-LO interaction
O.Werz	design of study, writing the manuscript
U. Garscha	design of study and experiments, writing the manuscript



Jena, den 25.06.2015 Prof. Dr. Oliver Werz

APPENDIX 5: EIGENSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich, dass mir die Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena bekannt ist, ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind.

Ich versichere, dass ich die Hilfe eines Promotionsberaters nicht in Anspruch genommen habe und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

Die vorliegende Dissertation wurde von mir bei keiner bisherigen Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht. Weiterhin versichere ich, dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Universität als Dissertation eingereicht habe.

Jena, den 25.06.2015 Jana Gerstmeier