Endogenous and exogenous modulation of 5-lipoxygenase

Impact of pregnancy, menstrual cycle and pharmacological inhibitors

Dissertation

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Abbreviations

5αHT 5α-dihydrotestosterone
AA .... arachidonic acid
ATP .... adenosine triphosphate
BCIP 5-bromo-4-chloro-3-indolylphosphate
BLT .... leukotriene B_4 receptor
BPB .... bromphenol blue
BSA, faf .... bovine serum albumin, essentially fatty acid free
CHX .... cycloheximide
CLP .... coactosin-like protein
COX .... cyclooxygenase
cPLA$_{2\alpha}$ .... cytosolic phospholipase $A_{2\alpha}$
cys-LT .... cysteinyl-leukotriene
CysLT$_{1/2}$ .... cysteinyl-leukotriene receptor 1/2
DAG .... diacylglyceride
DCF-DA .... 2',7'-dichlorofluorescence-diacetate
D-PBS .... dulbecco's phosphate-buffered saline
DPI .... diphenylene iodonium
DPPH 2,2-diphenyl-1-picrylhydrazyl
DTT .... dithiothreitol
EIA .... enzyme immunoassay
ERK .... extracellular-signal regulated kinase
FCS .... fetal calf serum
FLAP .... 5-lipoxygenase activating protein
fMLP .... N-formyl-methionyl-leucyl-phenylalanine
GPCR .... G-protein coupled receptor
GPX .... selenium-dependent glutathione peroxidases
GSH .... glutathione
HCG .... human chorionic gonadotropin
H(p)ETE .... hydroxy(peroxy)eicosatetraenoic acid
IL .... interleukin
i.p. .... intraperitoneally
LB-medium ...... lysogeny broth medium
LO .... lipoxygenase
LPS .... lipopolysaccharide
LSM .... lymphocyte separation medium
LTA$_4$H .... leukotriene A_4 hydrolase
LTC$_4$S .... cysteinyl-leukotriene C_4 synthase
LTRA .... leukotriene receptor antagonist
LUV .... large unilamellar vesicles
MAPEG .... membrane-associated proteins in eicosanoid and glutathione metabolism
MAPK .... mitogen-activated protein kinase

MCH .... mean cell hemoglobin
MCHC .... mean cell hemoglobin concentration
MCV .... mean cell volume
MK-2/3 .... mitogen-activated protein kinase activated protein kinase-2/-3
MIF .... macrophage inhibitory factor
MLV .... multilamellar vesicles
mPGES .... microsomal prostaglandin synthase
MTT .... 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH .... nicotinamide adenine dinucleotide phosphate-oxidase
NBT .... nitro blue tetrazolium
OAG 1-oleoyl-2-acetylglycerol
PAPC 1-palmitoyl-2-arachidonyl-*n*-glycero-3-phosphocholine
PBMC .... peripheral blood mononuclear cell
PC .... phosphatidylcholine
PDB .... protein data bank
PG .... prostaglandin
PG buffer .... D-PBS buffer containing 1 mg/ml glucose
PGC buffer .... PG buffer containing 1 mM $CaCl_2$
PKA .... protein kinase A
PLD/PA-P .... phospholipase D and phosphatidic acid phosphatase
PMA phorbol-12-myristate-13-acetate
PMNL .... polymorphnuclear leukocytes
PMSF .... phenylmethanesulphonyl fluoride
POG 1-palmitoyl-2-oleoyl-*sn*-glycerol
PPAR .... peroxisome proliferator-activated receptor
ROS .... reactive oxygen species
SAR .... structure activity relationships
SDS .... sodium dodecyl sulfate
SDS-PAGE .... SDS-polyacrylamide gel electrophoresis
SLE .... systemic lupus erythematosus
STI .... soybean trypsin inhibitor
TBS .... tris buffered saline
TEA .... triethanolamine
TFA .... trifluoroacetic acid
TGF .... transforming growth factor
Th cell .... T helper cell
TM .... transmembrane
TNF .... tumour necrosis factor
Tris .... trimethanolamine
TX .... thromboxane

Summary

This thesis comprises two parts both dealing with the modulation of 5-lipoxygenase (5-LO) the enzyme responsible for the synthesis of pro-inflammatory leukotrienes (LT). The first aim was to investigate if pregnancy influences LT formation and if so, to elucidate the underlying mechanisms. This was addressed by the use of blood assays in which the cells are in their physiologic environment as well as isolated cells and plasma preparations. Indeed, it was shown that pregnancy increases LT formation in blood and a concept of factors acting in synergism or in opposite directions was elaborated. (I) increased numbers of cells that are responsible for LT synthesis in blood from pregnant females ("plus effect"); (II) impaired LT formation capacity of isolated granulocytes from pregnant females ("minus effect"); (III) higher capability to form LTs of cells resuspended in plasma of pregnant females ("plus effect"). In coherence with published results by other groups concerning the role of LTs in parturition and the expression of LO enzymes in intrauterine tissues (Durn et al., 2010; Jian et al., 2013) the results of this thesis suggest that LTs might be involved in the immune regulation during pregnancy. Although it is unclear so far if LT formation is a modulatory part of the maternal immune system during pregnancy or rather a consequence of the ongoing changes. The results further call for a reevaluation of the application of LTRAs for asthma treatment of pregnant asthmatic patients.
Besides pregnancy also the female menstrual cycle was studied in respect of LT and eicosanoid formation. Revealing opposing trends of LTs and 12-HHT formation in luteal and follicular phase which might be related to the occurence of premenstrual asthma (Rao et al., 2013) and risk of myocardial infarction in the early follicular phase (Mukamal et al., 2002).

The second aim was to identify new 5-LO inhibitors and to characterize their molecular mode of inhibition as well as their selectivity. For this, the naturally occurring dihydrobenzoquinone embelin and related synthetic derivatives were studied. On the biological part, isolated 5-LO, intact cell and whole blood assays were applied. The obtained results at the isolated enzymes were further evaluated by the usage of computer-aided molecular docking studies. In this thesis, embelin was identified as potent inhibitor of both 5-LO and microsomal prostaglandin synthase 1 (mPGES-1). Embelin efficiently blocked LT formation in cellular models of human neutrophils and monocytes applying various stimuli. Related targets (12- and 15-LO, COX-1 and -2, $cPLA_2$) were not inhibited by embelin. Docking simulations by Dr. Daniela Schuster (University of Innsbruck, Austria) suggested concrete binding poses at the active sites of both 5-LO and mPGES-1. However, the interaction between embelin and Tyr-181 was not verified by studies with the 5-LO Tyr181A mutant. 5-LO inhibition by embelin was reversible, unaffected by increasing substrate amounts and Triton X-100, and did not correlate with its proposed antioxidant properties. Since 5-LO and mPGES-1 derived eicosanoids play roles in inflammation and cancer, the interference of embelin with these enzymes may contribute to its biological effects

observed in various animal models. Besides, RF-Id a related synthetic compound, was studied in regard to its molecular mode of 5-LO inhibition. RF-Id consistently blocked LT formation in cellular and blood assays and also showed anti-inflammatory activity *in vivo*. Mechanistically, RF-Id inhibits 5-LO in a nonredox fashion by discrete molecular interactions within the 5-LO active site. The latter were again studied by docking simulations. During the parallel research of embelin and RF-Id and their underlying mechanisms of inhibition, the question was addressed if benzoquinones are redox-type inhibitors. The results show that the inhibitory mode is not necessarily a redox-type inhibition but rather depends on the overall lipophilicity of the compound and the distinct interaction partners within the catalytic center of 5-LO. In total 31 hydroxybenzoquinones deduced from embelin were studied in this thesis. The compounds were synthesized by Dr. Rosanna Filosa (University of Salerno, Italy). The series comprises (I) 2,5-dihydroxylated 1,4-benzoquinones, (II) 2-hydroxy-5-methoxy-1,4-benzoquinones, (III) 2,5-methoxy-1,4-benzoquinones and (IV) 4,5-methoxy-1,2-benzoquinones which were modified in position 3 of the backbone with alkyl or prenyl chains. SARs revealed distinct features for potent inhibition of 5-LO in cell-free and cellular assays. At the isolated enzyme 2,5-dihydroxy-1,4-benzoquinone and 4,5-methoxy-1,2-benzoquinone backbones were superior over 2-hydroxy-5-methoxy-1,4-benzoquinones and 2,5-methoxy-1,4-benzoquinones. In the cellular assay, the potency was as follows: IV $>$ III $>$ II $>$ I. IC_{50} values for the most potent compounds were in the nanomolar range (0.03 to 0.06 μM). Furthermore, the degree of methylation of the hydroxyl groups determines the efficiency in the blood assay. Double methylation predominantly inhibits LT formation in blood ($IC_{50} = 3$ μM for compound 21). In cell-free as well as cellular assays, lipophilicity parallels with potency. Thus, chain lengths of 10 to 12 carbon atoms were optimal whereas shorter (C4, C6) or longer (C16) chains were detrimental. Similarly, the prenyl chain with three isoprene moieties was superior over two. The most potent ortho-quinone was subjected to detailed studies similarly to embelin and RF-Id. In summary, a potent candidate ($IC_{50} = 0.03$ μM, A23187) is presented with promising results in blood assays.

In conclusion, in the second part of the thesis naturally derived embelin was identified as potent inhibitor of 5-LO and mPGES-1. Embelin, RF-Id and the ortho-quinone 29 were studied in detail in respect to their molecular mode of inhibition and qualification as new drug candidates. All three compounds represent potent and selective inhibitors of 5-LO and in case of embelin also of mPGES-1.

Zusammenfassung

Die vorliegende Arbeit beinhaltet zwei Teile, die sich mit der Modulation der 5-Lipoxygenase (5-LO), dem Enzym, das für die Synthese von proinflammatorischen Leukotrienen (LT) verantwortlich ist, auseinandersetzen. Das erste Ziel (endogene Modulatoren) waren Untersuchungen darüber, ob eine Schwangerschaft die LT Bildung beeinflusst und falls dies der Fall sein sollte, die zu grunde liegenden Mechanismen aufzuklären. Um diese Frage zu beantworten, wurden sowohl Experimente mit Vollblut durchgeführt, in dem sich die Zellen in ihrer physiologischen Umgebung befinden, als auch Zellen und Plasma isoliert betrachtet. Es konnte gezeigt werden, dass während der Schwangerschaft die Leukotrienspiegel im Blut erhöht sind. Die erhöhte LT-Bildung setzt sich aus den folgenden Kompontenten zusammen, die entweder synergistisch oder gegensätzlich wirken: (I) erhöhte Mengen an Blutzellen, die für die LT-Synthese im Blut von Schwangeren verantwortlich sind ("Plus Effekt"); (II) verminderte LT-Bildung in isolierten Granulocyten von Schwangeren („Minus Effekt"); (III) höheres LT-Bildungsvermögen in Zellen, die in Plasma von Schwangeren resuspendiert wurden („Plus Effekt"). In Übereinstimmung mit veröffentlichten Daten von anderen Arbeitsgruppen bezüglich der Rolle der LT während der Geburt und der Expression von Lipoxygenasen in intrauterinen Geweben (Durn et al., 2010; Jian et al., 2013), legen die Ergebnisse dieser Arbeit nahe, dass LT in die Immunregulation während der Schwangerschaft eingebunden sind. Bisher ist jedoch unklar, ob die LT-Bildung während der Schwangerschaft ein modulatorischer Teil der Regulation des Immunsystems der Schwangeren ist oder eine Konsequenz aus den Veränderungen, die sich durch eine Schwangerschaft ergeben. Weiterhin fordern die Ergebnisse zu einer erneuten Überprüfung der Anwendung von Leukotrien-Rezeptor Antagonisten in der Asthma-Therapie während der Schwangerschaft auf. Neben der Schwangerschaft wurde auch der weibliche Menstruationszyklus hinsichtlich der LT- und Eikosanoid-Bildung untersucht. Es zeigte sich, dass LT- und 12-HHT-Bildung in der lutealen und follikulären Phase gegenläufig waren. Dies könnte im Zusammenhang mit dem Auftreten eines prämenstruellen Asthmas (Rao et al., 2013) sowie des erhöhten Risikos eines Myokard-Infarktes in der frühen follikulären Phase stehen (Mukamal et al., 2002).

Das zweite Ziel war die Identifizierung von neuen 5-LO Inhibitoren (exogene Modulatoren) und ihre inhibitorische Wirkung hinsichtlich des molekularen Mechanismus und der Selektivität zu untersuchen. Hierzu wurden der Naturstoff Embelin, ein Dihydroxybenzochinon und verwandte synthetische Derivate untersucht. Im biologischen Teil wurden Versuche mit isolierter 5-LO, intakte Zellen sowie Vollblut angewandt. Die Ergebnisse am isolierten Enzym wurden mit Hilfe von Computer gestützten molekularen Dockingstudien evaluiert. In dieser Arbeit, wurde Embelin als potenter Inhibitor der 5-LO und der microsomalen Prostaglandinsynthase-1 (mPGES-1) identifiziert. Embelin unterdrückte effizient die LT-Bildung in Zellmodellen mit humanen Neutrophilen und Monozyten, die mit verschiedenen Stimuli behandelt wurden. Verwandte Targets (12- und 15-LO, COX-1 und -2, $cPLA_2$) wurden nicht von Embelin gehemmt. Die Docking Simulationen von Dr. Daniela Schuster (Univer-

sität Innsbruck, Österreich) weisen auf konkrete Bindungsstellen im aktiven Zentrum von Embelin sowohl in der 5-LO als auch in der mPGES-1 hin. Jedoch konnte die Interaktion zwischen Embelin und Tyr-181 nicht anhand von Studien mit mutierter 5-LO bestätigt werden. Die 5-LO Inhibition durch Embelin war reversibel, unabhängig von verschiedenen Substratkonzentrationen und Triton X-100, und korrelierte nicht mit den beobachteten antioxidativen Eigenschaften der Substanz. Da die von 5-LO und mPGES-1 gebildeten Eikosanoide eine Rolle in der Entstehung von Entzündungen und Krebs spielen, kann die Interaktion von Embelin mit diesen Enzymen zum Verständnis der biologischen Effekte beitragen, die in verschiedenen Tiermodellen gefunden wurden. Daneben wurde RF-Id, eine Embelin strukturell verwandte synthetische Substanz, im Bezug auf den molekularen Hemmmechanismus an der 5-LO untersucht. RF-Id hemmte die LT-Bildung in zellulären Experimenten, im Blut und zeigte entzündungshemmende Eigenschaften *in vivo*. Mechanistisch hemmte RF-Id die 5-LO nach dem Nonredox-Typ mit bestimmten molekularen Interaktionen im katalytischen Zentrum der 5-LO. Dies wurde wieder mit Hilfe von Docking Simulationen untersucht. Während den parallelen Untersuchungen zu Embelin und RF-Id und den zugrunde liegenden Inhibitionsmechanismen kam die Frage auf, ob Benzochinone allgemein als redoxaktive Inhibitoren zu betrachten sind. Ergebnisse zeigten, dass der Mechanismus der Inhibition nicht ungedingt einem Redox-Typ folgt, jedoch stärker abhängig von der gesamten Lipophilie der Substanz, sowie der Interaktionspartner im katalytischen Zentrum der 5-LO ist. Es wurden weitere 31 von Embelin abgeleitete Hydroxybenzochinone in dieser Arbeit untersucht. Die Substanzen wurden von Dr. Rosanna Filosa (Universität Salerno, Italien) synthetisiert. Die Serie besteht aus (I) 2,5-dihydroxylierten 1,4-Benzochinonen, (II) 2-Hydroxy-5-methoxy-1,4-benzochinonen, (III) 2,5-Methoxy-1,4-benzochinonen und (IV) 4,5-Methoxy-1,2-benzochinonen, die jeweils in Position 3 des Grundgerüsts mit Alkyl- oder Prenylketten modifiziert sind. Die Strukturwirkbeziehungen zeigten klar abgegrenzte Eigenschaften im Bezug auf die potente Inhibition der Substanzen in zellfreien und in zellulären Assays. Am isolierten Enzym waren 2,5-Dihydroxybenzochinone und 4,5-Methoxy-1,2-dihydroxybenzochinone den 2-Hydroxy-5-methoxy-1,4-benzochinonen und 2,5-Methoxy-1,4-benzochinonen überlegen. Im zellulären Assay, war die Potenz wie folgt: IV $>$ III $>$ II $>$ I. IC_{50} Werte für die potentesten Substanzen waren im nanomolaren Bereich (0.03 μM bis 0.06 μM). Weiterhin, bestimmte der Grad an Methylierung der Hydroxy-Gruppen die Effizienz im Vollblut-Versuch. Zweifach Methylierung hemmte die LT-Bildung im Blut am stärksten ($IC_{50} = 3$ μM für Substanz 21). In zellfreien, sowie zellulären Versuchen war die Potenz abhängig von der Lipophilie der Substanzen. Dabei lag bei Kettenlängen zwischen 10 und 12 C-Atomen das Optimum, während kürzere Ketten von 4 und 6 C-Atomen oder längere von 16 C-Atomen nachteilig waren. Genauso waren bei prenylierten Ketten drei Isopren-Einheiten denjenigen mit zwei Einheiten überlegen. Das am stärksten wirksame *Ortho*-Benzochinon wurde detallierten Untersuchungen unterworfen wie zuvor schon Embelin und RF-Id. Es zeigte sich hier ein potenter Wirkstoffkandidat ($IC_{50} = 0.03$ μM) mit vielversprechenden Ergebnissen im Vollblutexperiment.

Zusammenfassend wurde im zweiten Teil der Arbeit der Naturstoff Embelin als potenter Inhibitor der 5-LO und der mPGES-1 identifiziert. Embelin, RF-Id und das *Ortho*-Benzochinon 29 wurden hinsichtlich ihres molekularen Wirkmechanismus und der Eignung als neue Wirkstoffkandidaten untersucht. Alle drei Substanzen erwiesen sich als potente und selektive Inhibitoren der 5-LO und im Falle von Embelin auch der mPGES-1.

1 Introduction

1.1 The immune system, inflammation and arachidonic acid-derived modulators

Inflammation is the answer of the immune system towards unanticipated events (e.g. pathogen invasion, toxins, irritants, trauma of tissue) in the body. Chronic inflammation is the dysbalance between the inflammation inducing and the resolving arm of the immune system. At the onset of inflammation, chemo-attraction of immune cells towards affected tissues takes places. A new paradigm was raised, the "lipid-cytokine-chemokine cascade" (Fig. 1.1), which describes the chronology of events leading to recruitment of immune cells to inflammatory sites. Interestingly, lipids are discussed to be the early signal (Sadik and Luster, 2012). These pro-inflammatory fast reacting mediators are quickly de-novo synthesized, which is more rapid than the transcription and translation process needed for proteins (e.g. interleukins (IL)) (Muller et al., 2009). This was, for example, demonstrated in a murine model of acute asthma where the leukotriene B_4 (LTB_4)/leukotriene B_4 receptor (BLT_1) interaction is involved in granulocyte recruitment during early but not late events (Medoff et al., 2006).

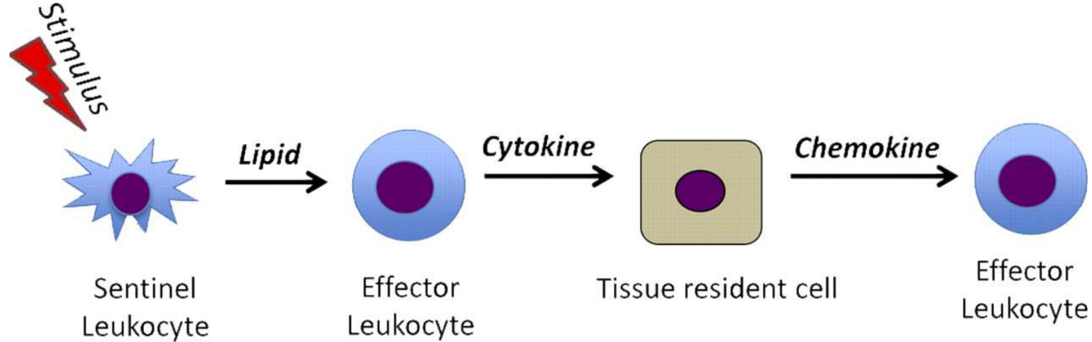

Figure 1.1: The "lipid-cytokine-chemokine cascade" (according to (Sadik and Luster, 2012)).

Since the beginning of arachidonic acid (AA) research there has been a boost in new findings. This C_{20} fatty acid is converted into numerous lipid mediators, each concerting individual effects in the human body. There are "pro-inflammatory" (e.g. leukotrienes, prostaglandins) as well as "anti-inflammatory" (e.g. resolvins, protectins, lipoxins, maresins) mediators, which are formed at distinct time points within an inflammatory process (for review see (Serhan and Petasis, 2011)).

Leukotrienes (LT) are formed from AA by catalysis of 5-lipoxygenase (LO). Different

roles of LTs in the pathogenesis of allergic and autoimmune diseases (i.e. asthma, systemic lupus erythematosus (SLE), rheumatoid arthritis, inflammatory bowel disease), arteriosclerosis as well as cancer are established (for review see (Haeggström et al., 2010)). Recently, disparities in the amount of LTs formed by neutrophils, monocytes and in the blood from males and females were found which support the sex-bias present in inflammatory diseases (Pergola et al., 2008, 2011). Search for new and potent inhibitors of the AA pathway does not solely comprise the idea to find therapeutics to cure inflammatory diseases, but also aims at the understanding of the regulation of lipoxygenases at molecular level.

This thesis is divided into two major parts. First, endogenous modulators of lipoxygenases will be discussed with a special focus on the impact of pregnancy on LT formation. Second, the influence of exogenous modulators – new inhibitors of 5-lipoxygenase and their molecular interactions – will be addressed.

1.2 The arachidonic metabolic network

AA is stored, ready to be used in the membranes of cells and cleaved from the *sn*2 position of the glycerol backbone of phospholipids by phospholipases, mainly by cytosolic phospholipase A_2 ($cPLA_2$) (Clark et al., 1991; Uozumi et al., 1997). AA is metabolized into various lipid mediators as shown in (Fig. 1.2). Besides AA, there are also other related unsaturated fatty acids which use identical enzyme pathways. Eicosanoids unfold their action via G-protein coupled receptors (GPCR) and nuclear receptors, mainly via peroxisome proliferator-activated receptor (PPAR) (Back et al., 2011). The vast variety of eicosanoid mediators are classified into three groups: lipoxygenase (LO)-, cyclooxygenase (COX) - and CYP450-derived substances.

1.2.1 Lipoxygenases

LOs comprise a family of dioxygenases which are found in plants, marine organisms and mammalians. Many mechanistic studies are based on soybean LO-1 (a 15-LO) (Kuhn and Thiele, 1999). In humans, six LOs are known and categorized according to the position of their attack at AA (Brash, 1999). If there is more than one LO leading to the same product in a species, the tissue where the LO is expressed is also included in the designation. For example, there are 3 types of 12-LOs known in mammalians: platelet-, leukocyte- and epidermal-type 12-LO (Brash, 1999).

1.2.2 Human 5-Lipoxygenase

LT research started in the late 1970s as a continuation of the work on AA-derived prostaglandins (Samuelsson, 2000). Rabbit peritoneal as well as human blood derived polymorphnuclear leukocytes (PMNL) form LTs following stimulation with Ca^{2+}-mobilizing agent A23187 alone or together with AA (Borgeat et al., 1976; Borgeat and Samuelsson, 1979a). The elucidation of the components of the "slow-reacting substance of anaphylaxis", namely cysteinyl-LTs (cys-LTs), was part of the research interests at that time (Murphy et al., 1979). The name "leukotrienes" was determined by the cellular source of the metabolites ("leukocytes") as well as their chemical structure (conjugated trienes) (Samuelsson et al., 1979). 5-LO was identified as the LT-forming enzyme in PMNL (Borgeat et al., 1976; Rouzer and Samuelsson, 1985).

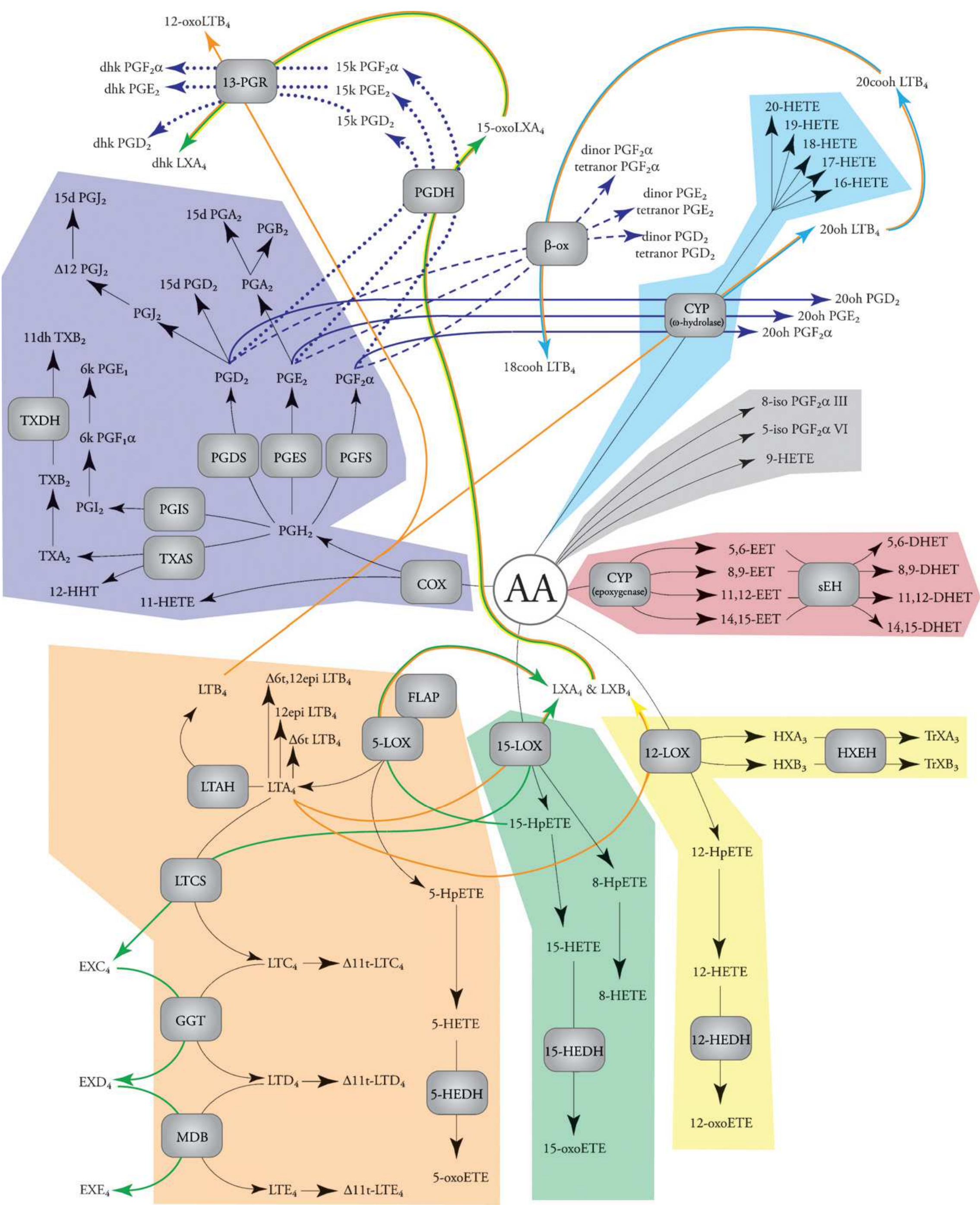

Figure 1.2: AA derived products. Overview of the various pathways (according to (Buczynski et al., 2009)).

5-LO catalysis and LT biosynthesis

The mechanism of AA conversion was elucidated step by step studying cellular models (Borgeat and Samuelsson, 1979b,c). The 5-LO enzyme combines two enzymatic functions (Shimizu et al., 1984): (A) insertion of molecular oxygen by a dioxygenase activity and (B) epoxide formation by an epoxide synthase activity (Rouzer et al., 1986).

5(S)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HPETE) is formed from AA by stereochemically controlled subtraction of the hydrogen in position C-7 (Fig. 1.3, I), radical rearrangement (Fig. 1.3, II) and insertion of molecular oxygen at C-5 (Fig. 1.3, III) (Corey and Lansbury, 1983).

Figure 1.3: Mechanism of 5-HPETE synthesis by 5-LO. (I) stereoselective abstraction of the pro-S hydrogen at C-7; (II) radical rearrangement; (III) insertion of molecular oxygen; (IV) generation of the peroxy anion; (V) protonation.

The epoxide synthase function of 5-LO converts 5-HPETE either into the unstable epoxide 5,6-epoxy-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (LTA_4) (Fig. 1.4, Ia-III) or via reduction into the corresponding alcohol 5(S)-hydro-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HETE) (Fig. 1.4, Ib) (Maas et al., 1982). The ratio of LTA_4 versus 5-HPETE depends on the assay conditions applied. High substrate AA concentrations lead to lower ratios while higher LTA_4 levels are formed in presence of 5-lipoxygenase activating protein (FLAP), coactosin-like protein (CLP) and 5-LO binding to membranes (Abramovitz et al., 1993; Hill et al., 1992; Rakonjac et al., 2006). In preparations of purified 5-LO enzyme, LTA_4 decomposes into two isomers (6-*trans* LTB_4 and 6-*trans*-12-epi LTB_4) (Borgeat and Samuelsson, 1979b). 5-HETE is oxidized to the eosinophil chemoattractant 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) by 5-hydroxyeicosanoid dehydrogenase (5-HEDH) (Powell et al., 1992; Powell and Rokach, 2005). Additionally, 5-HETE is converted into pro-resolving lipoxins by the combination of 5-, 12- and 15-LO via transcellular metabolism (Powell et al., 1992; Powell and Rokach, 2005; Serhan et al., 2008).

Figure 1.4: Mechanism of LTA_4 synthesis by the epoxide synthase activity of 5-LO and further transformation of LTA_4. (Ia) abstraction of the pro-R hydrogen at C-10; (Ib) reduction of the peroxide; (II) radical rearrangement; (III) dehydration of the hydroperoxide and formation of the epoxide. (IV) Hydrolysis of the 5,6-epoxide LTA_4 to the dihydroxy LTB_4 by the LTA_4 hydrolase. (V) Synthesis of LTC_4, LTD_4 and LTE_4 by the LTC_4 synthase, γ-glutamyltransferase (LTD_4) and dipeptidase (LTE_4).

The cell type determines the fate of the unstable 5,6-epoxide LTA_4. Ubiquitously expressed soluble LTA_4 hydrolase (LTA_4H) forms LTB_4 by stereospecific hydrolysis of the epoxide LTA_4 (Fig. 1.4, IV) (Rådmark et al., 1984). In addition to the hydrolase activity, LTA_4H also carries an aminopeptidase function (Haeggström et al., 1990). The crystal structure of LTA_4H (in complex with the inhibitor bestatin) has been known since 2001 (Thunnissen et al., 2001). The catalytic domain of LTA_4H contains a zinc ion, which is stabilized by His-295, His-299 and Glu-318 (Medina et al., 1991). The Zn^{2+} interacts with the expoxide of LTA_4 by formation of a carbocation and the epoxide is cleaved at C-6. Asp-375 polarizes a water molecule, which is inserted at C-12 under stereospecific control (Thunnissen et al., 2001).

In monocytes, dendritic cells and mast cells LTA_4 can be converted into cysLTs by LTC_4 synthase (LTC_4S) (Fig. 1.4, V). This glutathione-S-transferase attaches glutathione (GSH) to LTA_4 and LTC_4 is formed (Yoshimoto et al., 1985). The stepwise cleavage of the glutathione tripeptide leads to LTD_4 and subsequently to LTE_4. For LTD_4, the γ-glutamyl residue is removed by a γ-glutamyltransferase (Fig. 1.4, V). Likewise, the glycine is eliminated by dipeptidases in case of LTE_4 (Fig. 1.4, V) (Anderson et al., 1982; Lee et al., 1983). LTC_4 synthase belongs to the family of "membrane-associated proteins in eicosanoid and glutathione metabolism" (MAPEG) and was crystallized in 2007 (Ago et al., 2007; Martinez Molina et al., 2007). Experiments in knockout mice show that LTC_4 synthase is the most important enzyme for the synthesis of cysLTs (Kanaoka et al., 2001).

The crystal structure of 5-LO

The human 5-LO is a monomeric protein (673 amino acids) with a calculated mass of 78 kDa (Matsumoto et al., 1988). The recently published crystal structure (2.4 Å resolution) of a stable 5-LO mutant replaces the models based on the crystal

structure of rabbit reticulocyte-type 15-LO (Gilbert et al., 2011; Gillmor et al., 1997). In order to overcome the instability of the wild-type 5-LO, several sequences were exchanged in the stable 5-LO mutant. For example, the lysine-rich sequence (K653KK655), which positions the C-terminus, was exchanged by Glu-Asn-Leu (Gilbert et al., 2011). As for all LOs, 5-LO consists out of two domains: the N-terminal "C2-like (residues 1-114)" and the catalytic domain (residues 121-673). The "C2-like" domain is involved in the binding of 5-LO to membranes via Ca^{2+} (Hammarberg et al., 2000). The catalytic domain is mainly formed by α-helices and exhibits the non-heme catalytic iron (Fig. 1.5).

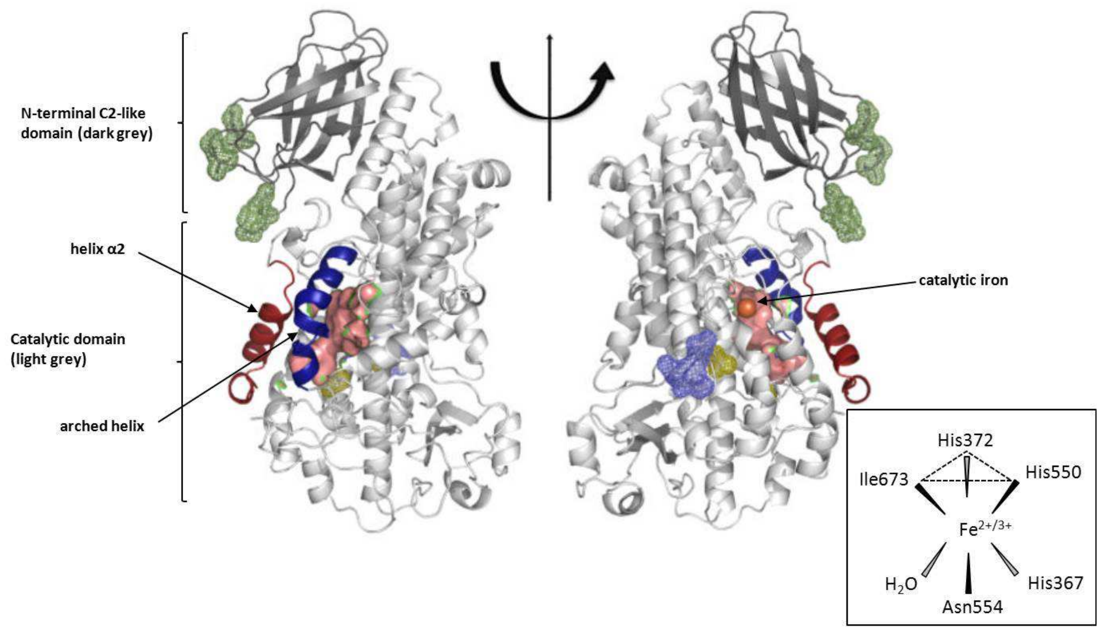

Figure 1.5: The structure of stable 5-LO according to (Gilbert et al., 2011) with modifications. The structure is shown in two different perspectives by 180° rotation as indicated. Pink: cavity surface; shaded areas: mutations; green: putative membrane insertion residues; yellow: proximal cysteines; blue: KKK to ENL replacement. The box represents the ligands at the catalytic iron (modified after (Rådmark and Samuelsson, 2009).)

In accordance with the general lipoxygenase model, the iron is surrounded by five conserved ligands: three histidines (His-367, 372 and 550), one asparagine (Asn-554) and the C-terminal isoleucine (Ile-673) (Hammarberg et al., 1995; Percival and Ouellet, 1992; Zhang et al., 1993). H_2O forms the sixth ligand. His-372 and 550 as well as Ile-672 were discussed as permanent ligands meaning that their mutation was detrimental to activity and iron content of the enzyme (Percival and Ouellet, 1992; Zhang et al., 1993). His-367 and Asn-554 as flexible ligands are crucial for complete activity of 5-LO but not for iron binding (Hammarberg et al., 1995; Zhang et al., 1993).
An arched helix, which contains a leucine (Leu-414) at the zenith, masks the catalytic iron. Together with helix $\alpha 2$, the elongated cavity is formed (Gilbert et al., 2011). Compared to 8R-LO and 15-LO, the helix $\alpha 2$ is shorter in the stable 5-LO (three compared to six or seven turns) and has a different orientation (Fig. 1.6) (Gilbert et al., 2011).

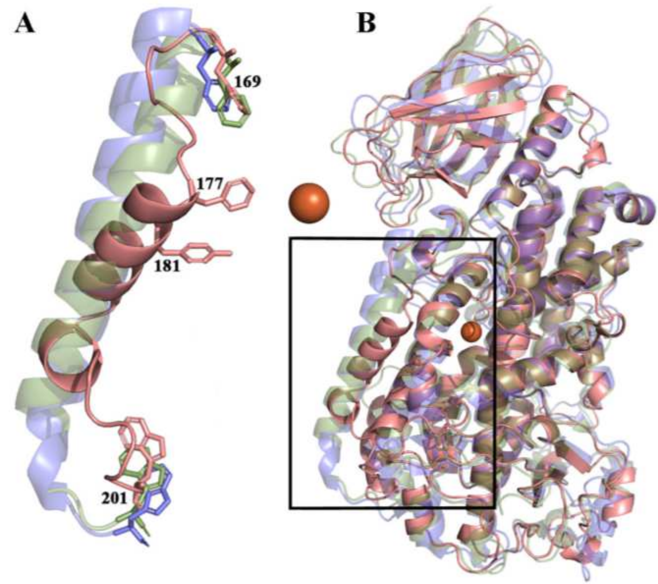

Figure 1.6: Stable 5-LO (red) and the orientation of the helix $\alpha 2$ compared to 8R-LO (green) and 15-LO (blue) (Gilbert et al., 2011). The box shows the enlarged region in (A) which comprises the helix $\alpha 2$. The catalytic iron is orange. The overlay of the three structures (5-LO, 8R-LO and 15-LO) is shown in (B).

The hydrophobic chains of Leu-368, 373, 414 and 607 and of Ile-406 form the cavity in which the substrate AA is positioned in the catalytic domain leading to the correct orientation of the pentadiene. This positioning is conserved among the lipoxygenases (Neau et al., 2009). Several amino acids are specific for the sequence of 5-LO such as Tyr-181, Ala-603, Ala-606, His-600 and Thr-364. Interestingly, Phe-177 and Tyr-181 form a so-called FY cork which closes the entrance of the cavity. The formation of the lid is further supported by Ala-603, Ala-606 and Trp-599 (Gilbert et al., 2011). This cork is found only in 5-LO and not in 8R- or 15-LO. However, up to now it is not clear how the cork opens and how AA enters the catalytic cavity (Gilbert et al., 2011).

The redox-cycle of 5-LO

The presence of the catalytic non-heme iron is essential for 5-LO activity (Percival, 1991). For catalysis, the iron is first brought from the inactivated (Fe^{2+}, ferrous) to the activated state (Fe^{3+}, ferric) (Chasteen et al., 1993; Hammarberg et al., 2001). This reaction is reversible (Chasteen et al., 1993) and suggests a cycling of the iron between the two redox stages. The purified 5-LO enzyme is activated in presence of low concentrations fatty acid hydroperoxides (5-HPETE, 12-HPETE or 13-hydroperoxyoctadecadienoic acid (HPOD)), which are needed for optimal enzyme kinetics without an initial lag phase (Riendeau et al., 1989; Rouzer et al., 1986). In excess, hydroperoxides lead to inactivation of 5-LO (Aharony and Stein, 1986). Fig. 1.7 shows the presumed catalytic cycle of 5-LO (Werz and Steinhilber, 2005a). The redox regulation of the catalytic cycle is important for the understanding of the inhibitory mode of redox-type 5-LO inhibitors that reduce the iron and keep it in the inactivated state (Rouzer et al., 1991).

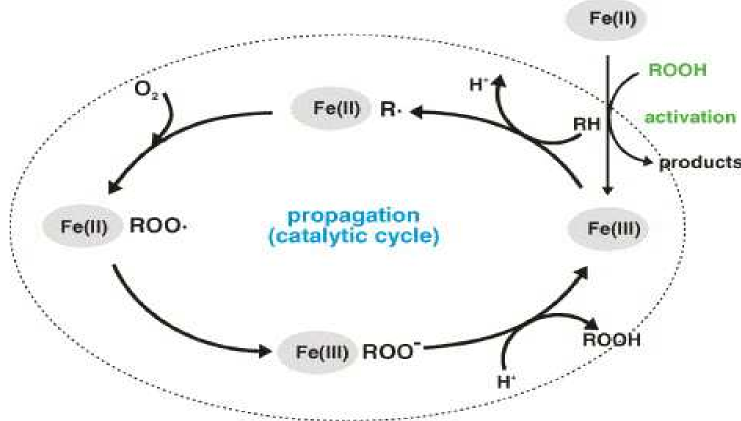

Figure 1.7: Regulation of the catalytic cycle of 5-LO (according to (Werz and Steinhilber, 2005a)). ROOH = fatty acyl hydroperoxides; RH = substrate of 5-LO.

Regulation of 5-LO activity

Several factors were shown to activate 5-LO. While some factors are essential for the catalytic activity of the isolated enzyme, some are only crucial for LT biosynthesis in cells (Fig.1.8). Product formation of the isolated 5-LO is influenced by the availability of Ca^{2+}, AA, hydroperoxides, phospholipids, triglycerides and adenosine triphosphate (ATP). In cells, 5-LO activity is driven by the intracellular localization and the membrane binding of 5-LO, phosphorylation events, the substrate supply by phospholipases, the interaction with intracellular proteins such as coactosin-like protein (CLP) or 5-lipoxygenase activating protein (FLAP) and the cellular redox tone (for review see (Werz and Steinhilber, 2005a)).

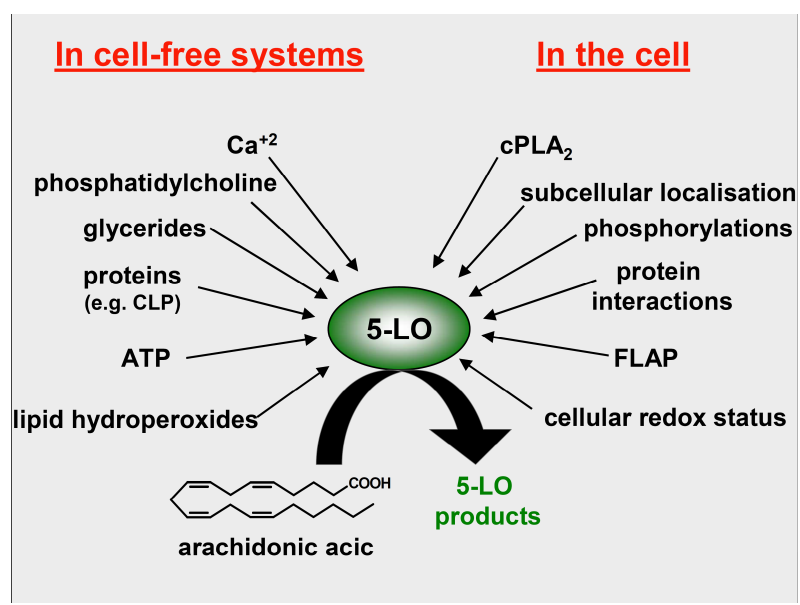

Figure 1.8: Factors that regulate 5-LO catalysis in cell-free and cellular systems (Werz and Steinhilber, 2005a).

Calcium

Calcium ions are assumed to bind to the N-terminal β-barrel C2-like domain of 5-LO, which is also found in other enzymes like $cPLA_2$ or protein kinase C (Hammarberg et al., 2000). Site-directed mutagenesis revealed Asn-43, Asn-44 and Asn-46 as possible binding ligands situated in loop 2 of the N-terminal domain of 5-LO (Hammarberg et al., 2000). It was demonstrated that Ca^{2+} binds with an average stoichiometry of 2:1 per 5-LO enzyme molecule and a Kd of about 6 μM was estimated (Hammarberg and Rådmark, 1999). Binding of Ca^{2+} increases the hydrophobicity of the enzyme (Hammarberg and Rådmark, 1999) and also neutralizes the negative charge of the C2-like domain to a certain extent (Kulkarni et al., 2002). In the cell, Ca^{2+} seems to mediate the binding of 5-LO to membranes (Chen and Funk, 2001; Rouzer and Samuelsson, 1987). Therefore, Ca^{2+} is essential for the translocation to the nuclear membranes and the following interaction. Ca^{2+} binding might lead to conformational changes of 5-LO and subsequent positioning of the tryptophan residues (Trp-13, 75 and 102) to enable the interaction (Kulkarni et al., 2002). Addition of Ca^{2+} strongly increases product formation and velocity and reduces the lag phase as well as the Ks (Aharony and Stein, 1986). There are diverging reports on the amount of Ca^{2+} sufficient for the activation of 5-LO. Concentrations from 0.1 to 10 μM Ca^{2+} were found to concentration-dependently induce product formation from isolated 5-LO (Percival and Ouellet, 1992). In the neutrophil, it was shown that elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) as well as extracellular Ca^{2+} ($[Ca^{2+}]_{ex}$) are crucial for the induction of LT synthesis (Krump et al., 1995). Starting from about 150 nM $[Ca^{2+}]_i$, a linear dependency was observed in neutrophils stimulated with ionomycin, AA and extracellular Ca^{2+} in excess, up to the threshold level of $cPLA_2$ (350-400 nM) (Schatz-Munding et al., 1991).

Adenosine-triphosphate

Partially purified 5-LO from guinea pig and human PMNL showed a concentration-dependent increase of product formation by adenosine-triphosphate (ATP) (Ochi et al., 1983; Noguchi et al., 1996; Rouzer and Samuelsson, 1985). Various ATP-analogues such as ADP, AMP, cAMP, CTP etc. also enhanced product synthesis but to a smaller extent compared with ATP (Ochi et al., 1983). Non-hydrolysable γ-S-ATP induced product formation in a similar way as ATP, implying that ATP is not hydrolyzed during 5-LO catalysis (Noguchi et al., 1996)). Also for human leukocyte derived 5-LO, presence of ATP leads to maximal activity (Noguchi et al., 1996; Rouzer and Samuelsson, 1985). The stoichiometry was calculated to be 1:1 for ATP or ATP analogues (Zhang et al., 2000). Due to the reversible binding of ATP to 5-LO, ATP affinity column chromatography was applied for the purification of 5-LO (Denis et al., 1991; Furukawa et al., 1984). Additionally, ATP is discussed to increase 5-LO enzyme stability (Rådmark and Samuelsson, 2009).

Membranes, phospholipids, phosphatidylcholine and glycerides

Early research showed that membranes are crucial for the complete activity of 5-LO. Increased product formation was observed after addition of membrane fractions to purified 5-LO (Rouzer and Samuelsson, 1985; Rouzer et al., 1985). Phosphatidylcholine (PC) vesicles can substitute the membrane fractions and were shown to be a scaffold factor for 5-LO activity (Puustinen et al., 1988). Vesicles composed of related phospholipids did not increase 5-LO product formation capacity (Noguchi et al., 1994; Puustinen et al., 1988), although co-precipitation of 5-LO with phospha-

tidylethanolamine and -serine liposomes in presence of Ca^{2+} was described (Noguchi et al., 1994). Detailed studies concerning the lipid composition of membranes and 5-LO binding were conducted. In the cell, 5-LO translocates towards the nuclear envelope (Rouzer and Kargman, 1988). The reason for this is under debate. Two explanations might be possible. First, it was suggested that high amounts of PC in the nuclear membrane stimulate 5-LO activity (Kulkarni et al., 2002). Second, the membrane fluidity of the AA rich nuclear envelope might attract 5-LO (Pande et al., 2005). Membrane fluidity is increased by the grade of acyl chain unsaturation in *sn*-2 position of PCs. Interestingly, 5-LO activity as well as binding affinity was highest for 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine (PAPC) (Pande et al., 2005). 5-LO product formation was inhibited by inclusion of cholesterol into artificial membranes that reduced membrane fluidity (Pande et al., 2005). Also in a cellular assay, cholesterol sulfate inhibits LT formation (Aleksandrov et al., 2006).

In addition to phospholipids also glycerides like 1-oleoyl-2-acetylglycerol (OAG) and to a lower extent also 1-O-oleyl-*rac*-glycerol, 1,2-dioctanoyl-*sn*-glycerol and 1-O-hexadecyl-2-acetyl-*sn*-glycerol but not 1-stearoyl-2-arachidonoyl-*sn*-glycerol were discussed to increase 5-LO catalysis in absence of Ca^{2+} (Hornig et al., 2005). OAG binds directly to 5-LO via the same binding site as PC, which was shown by site-directed mutagenesis at Trp-13, 75 and 102 (Hornig et al., 2005; Kulkarni et al., 2002). It was shown that intracellular generation of diacylglyceride (DAG) is involved in 5-LO activation. Inhibition of the DAG forming pathway (phospholipase D and phosphatidic acid phosphatase (PLD/PA-P)) leads to decreased 5-LO product synthesis as well as translocation. Supplementation of OAG reversed this (Albert et al., 2008). Therefore, it was concluded that DAG formed via the PLD/PA-P pathway is a possible stimulatory mechanism of cellular 5-LO catalysis (Pergola et al., 2011). Interestingly, in monocytes from male donors DAG formation is reduced due to the inhibition of PLD by testosterone which results in lower LT biosynthesis compared to cells from female donors (Pergola et al., 2011).

Phosphorylation

Cellular stimulation with cell stress (e.g. osmotic shock, oxidative or chemical stress) inducing compounds or phorbol esters like phorbol-12-myristate-13-acetate (PMA) increase 5-LO product formation as well as translocation of the enzyme and parallels with the activation of kinases that are able to phosphorylate 5-LO (Werz et al., 2001a,b). Indeed, cellular LT formation and enzyme translocation are dependent on the phosphorylation of 5-LO. Diverse kinase inhibitors such as tyrosine kinase inhibitors, protein kinase C (PKC) inhibitor calphostin C, mitogen-activated protein kinase kinase 1 inhibitor PD98059 and U0126 and p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 interfered with LT synthesis (Boden et al., 2000; Lepley and Fitzpatrick, 1996; Lepley et al., 1996; Werz et al., 2000, 2001b). The 5-LO protein contains several motifs prominent for the attack by kinases. Out of these, three amino acids are known to be phosphorylated. Phosphorylation of Ser-271 and Ser-663 leads to activation of 5-LO (Werz, 2002; Werz et al., 2000, 2002b) while phosphorylation of Ser-523 causes its inactivation (Luo et al., 2004). At Ser-271 and Ser-663, 5-LO is phosphorylated by mitogen-activated protein kinase activated protein kinase-2/-3 (MK-2/3), extracellular-signal regulated kinase (ERK) or Ca^{2+}/calmodulin-dependent kinase II (Werz, 2002; Werz et al., 2000, 2002b). Phosphorylation leads to increased 5-LO activity mainly by inducing translocation of the

enzyme to nuclear membranes and not by intrinsic activation of the enzyme itself. The significance of the phosphorylation strongly depends on the type of stimulus used (Rådmark and Samuelsson, 2005). Stimuli causing slight increases of $[Ca^{2+}]_i$ seem to activate 5-LO by MK-2 and ERK catalyzed phosphorylation (Werz, 2002; Werz et al., 2002b). Phosphorylation at Ser-523 by PKA, however, suppresses 5-LO activity and interferes with 5-LO translocation to the nuclear membranes (Luo et al., 2005, 2004). Mutation of Ser-523 to the phosphate analogue Glu reduced 5-LO activity. Mutation of Ser-523 to Ala disables the phosphorylation of 5-LO by PKA (Luo et al., 2004). This provides the molecular basis for the observed suppression of LT synthesis by adenosine and cAMP which activate PKA (Flamand et al., 2002).

Protein interaction and proteins known to directly activate 5-LO
Interaction of 5-LO with proteins was first investigated by screening the human lung cDNA library with a two-hybrid approach. CLP, transforming growth factor (TGF) type β receptor-I-associated protein 1 and the ribonuclease III enzyme dicer were identified as 5-LO binding proteins (Provost et al., 1999). Furthermore, 5-LO associates with the growth factor receptor-bound protein 2, which is important for tyrosine kinase-mediated cell signaling (Lepley et al., 1996). CLP (142 amino acids, 17 kDa) is related to F-actin binding coactosin, which was first detected in Dictostyostelium discoideum (de Hostos et al., 1993; Provost et al., 1999). A 1:1 complex of 5-LO and CLP is formed (Provost et al., 2001). CLP stimulated total production formation (LTA_4 derived isomers, 5-HPETE and 5-HETE) of purified 5-LO in presence of Ca^{2+} and AA (Rakonjac et al., 2006). Trp-102 came up to be crucial for CLP/5-LO interaction as well as enzyme activity (Esser et al., 2010; Rakonjac et al., 2006). Besides PC also CLP is discussed as a scaffold factor or chaperone, supporting the structure of 5-LO. In this respect, CLP also reduced the non-turnover but not turnover inactivation of 5-LO (Esser et al., 2010).

5-Lipoxygenase-activating protein
5-Lipoxygenase-activating protein (FLAP) (18 kDa, 161 amino acids) was detected during the elucidation of two phenomena: the discovery of MK-886, a LT synthesis inhibitor that does not directly interfere with the 5-LO enzyme, and the observation that 5-LO transfected osteosarcoma cells exhibited LT formation capacity in cell homogenates but not in the entire cells stimulated with A23187 (Dixon et al., 1990; Miller et al., 1990). FLAP was grouped in the MAPEG family though it does not bind GSH nor has a known enzymatic activity (Bresell et al., 2005; Martinez Molina et al., 2008). Co-transfection of Sf9 cells with 5-LO and FLAP leads to higher LT formation capacity than transfection with 5-LO only. A higher ratio of 5-HPETE conversion to LTA_4 following stimulation with A23187 and AA is found (Abramovitz et al., 1993).

The exact role of FLAP in LT synthesis is still unknown. It was shown that FLAP binds $[^{125}I]$L-739,059, a photoaffinity analogue of AA. The binding was competed by AA and MK-886 (Mancini et al., 1993). In the cell, FLAP is thought to bind AA and to provide it to 5-LO. Thereby, FLAP increases the efficacy by 5-LO to utilize AA (Abramovitz et al., 1993). It was shown that the formation of the 5-LO/FLAP complex as well as the closeness of this association depends on the presence of AA (Bair et al., 2012). While 5-LO translocates upon stimulation, FLAP is constantly located at the nuclear envelope (Woods et al., 1993). After stimulation, 5-LO and FLAP are found in the same compartments of the nuclear membrane in close pro-

ximity (Woods et al., 1993). It is still unclear how 5-LO and FLAP interact on a molecular basis. However, it is reported that LTC_4S binds with distinct parts to FLAP as well as 5-LO (Mandal et al., 2004; Strid et al., 2009) and thus a multi-protein LT synthetic complex at the nuclear envelope is formed (Mandal et al., 2008).

Lipid hydroperoxides and the influence of the cellular redox tone
As described, the redox cycle is a crucial step in the catalytic reaction of 5-LO. Upon the addition of lipid hydroperoxides such as 5-HPETE, 12-HPETE, 15-HPETE or 13-HPODE, the iron is oxidized (Hammarberg et al., 2001) and the lag phase is reduced (Riendeau et al., 1989). Addition of GSH together with selenium-dependent glutathione peroxidases (GPX) or reducing agent dithiothreitol (DTT) to purified 5-LO preparations or cell homogenates reduces 5-LO activity due to reduction of lipid hydroperoxides, which are needed to activate 5-LO (Haurand and Flohe, 1988; Rouzer and Samuelsson, 1986). In the cell, GPX catalyzes reaction of GSH to form GSSG by in parallel reducing phospholipid hydroperoxides. In leukocytes, phospholipid hydroperoxide GPX (GPX-4) was shown to influence 5-LO activity by regulation of the cellular redox tone (Weitzel and Wendel, 1993). The GPX subtype that is responsible for this effect is dependent on the cell-type. In monocytic cells GPX-1 and in B-lymphocytes, RBL-2H3 and immature HL60 cells GPX-4 regulates the redox tone which influences 5-LO activity (Imai et al., 1998; Straif et al., 2000; Werz and Steinhilber, 1996). Depletion of GSH (e.g. by diamide) or inhibition of GPX in neutrophils increases 5-LO product formation (Hatzelmann et al., 1989). Interestingly, oxidative stress induces p38 MAPK activation and subsequent phosphorylation, translocation and stimulation of 5-LO catalytic activity. SB203580, a p38 MAPK inhibitor prevented this stress-induced activation (Werz et al., 2001a).

Subcellular localization
As indicated, subcellular localization is important for the catalytic capacity of 5-LO. This is accompanied by the formation of the LT synthesis enzyme metabolon (5-LO, $cPLA_2$, FLAP, LTC_4S) (Bair et al., 2012; Peters-Golden and Brock, 2001). Stimulation with A23187 leads to translocation of 5-LO and in parallel of $cPLA_2$ to the nuclear membranes (Pouliot et al., 1996). Two reasons for this pattern of subcellular organization are obvious: first, the availability of substrate AA and second, the interaction of 5-LO with upstream ($cPLA_2$) and downstream enzymes (LTC_4S). Interestingly, differential locales of 5-LO downstream enzymes are found as demonstrated in Fig. 1.9 and reviewed in (Newcomer and Gilbert, 2010). Note that LTA_4H is a soluble enzyme while LTC_4S is situated at the nuclear membrane. Depending on the cell-type 5-LO is located in the cytosol (peritoneal macrophages, monocytes, neutrophils, eosinophils) or both cytosol and nucleus (mast cells, alveolar macrophages) in resting cells (Peters-Golden and Brock, 2003). Localization of 5-LO parallels with differentiation of monocytes (cytosolic) to alveolar macrophages (nuclear) (Covin et al., 1998). Furthermore, following adhesion or recruitment of eosinophils and neutrophils to inflammatory sites, 5-LO is also imported into the nucleus (Brock et al., 1999, 1997). Though in the resting cells different location patterns were described, in all cells (except for eosinophils) nuclear localization leads to higher LT synthesis capacity than cytosolic localization. However, not only cell-type specific localization of 5-LO but also sex-specific disparities in unstimulated neutrophils were found. 5-LO is located in the cytosol as well as at the nuclear membrane in resting neutrophils from males but only in the cytosol in cells from females (Pergola et al.,

2008).

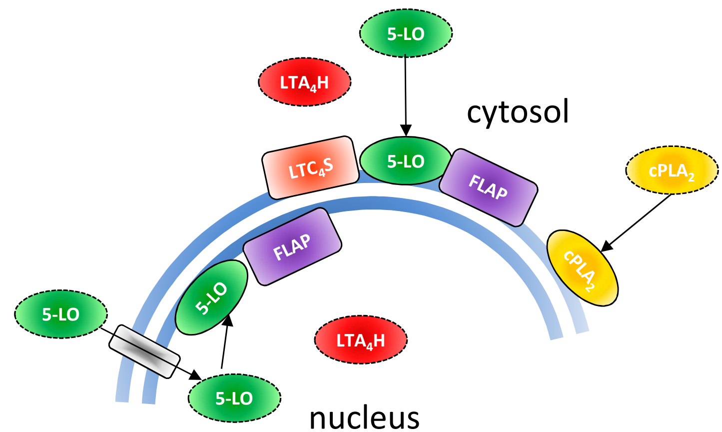

Figure 1.9: Enzyme localization of the LT synthesis metabolom at the nuclear membrane. 5-LO = 5-lipoxygenase; FLAP = 5-lipoxygenase activating protein; $cPLA_2$ = cytosolic phospholipase A_2; LTC_4S = LTC_4 synthase; LTA_4H = LTA_4 hydrolase. Cytosolic enzymes are marked by dashed lines; mobile enzymes are ovals and resident enzmyes are rectangles.

Cytosolic phospholipase A_2
In the cell, the liberation of AA from *sn*-2 position of phospholipids is the rate-limiting step in LT synthesis. Up to now about 15 PLA_2 groups are known which are subdivided in four main categories: secretory PLA_2, calcium-independent PLA_2, platelet activating factor acetyl hydrolase/oxidized lipid lipoprotein associated PLA_2 and cytosolic PLA_2($cPLA_2$) (Burke and Dennis, 2009; Leslie, 1997). $cPLA_2$ is unique in its preference for the cleavage of AA in position *sn*-2 of phospholipids (Clark et al., 1991). The crystal structure of the 85 kDa protein revealed a Ca^{2+} binding C2 and a catalytic domain. The catalytic center consists of a Ser-228/Asp-549 dyad. Interestingly, a lid closes the entrance to the catalytic domain in $cPLA_2$, which is suggested to be removed by conformational changes due to membrane binding (Dessen et al., 1999). Activation pathways of $cPLA_2$ parallel with that of 5-LO. Thus, an increase in Ca^{2+}, phosphorylation of $cPLA_2$ at Ser-505 as well as the translocation of the enzyme to nuclear membranes are crucial for complete catalytic activity (Glover et al., 1995; Lin et al., 1993; Schievella et al., 1995). Phosphorylation of $cPLA_2$ is sufficient for activation even at low Ca^{2+} levels. Conversely, at high Ca^{2+} levels (after stimulation with A23187) phosphorylation is not needed for AA release (Gijon et al., 2000). The relevance of $cPLA_2$ is demonstrated by $cPLA_2$ knock-out mice models in which LT and eicosanoid formation was completely suppressed and bronchial hyperactivity was not developed in asthma models (Bonventre et al., 1997; Uozumi et al., 1997; Uozumi and Shimizu, 2002).

Cellular stimulation of LT synthesis

In general, cellular LT synthesis is stimulated by Ca^{2+}-mobilizing agents such as ionophores (Bach and Brashler, 1974; Borgeat and Samuelsson, 1979a) and thapsigargin (Ohuchi et al., 1987; Wong et al., 1991) which activate both $cPLA_2$ and 5-LO. Combination of ionophor A23187 with AA leads to higher product formation compared to stimulation with each substance alone (Palmer and Salmon, 1983). Pathophysiological relevant agonists such as N-formyl-methionyl-leucyl-phenylalanine (fMLP) (Salari et al., 1985), platelet activating factor (Chilton et al., 1982) and C5a

(Clancy et al., 1983) cause low or no induction of LT formation compared to ionophors. Those naturally occurring stimuli do not lead to sufficient increase of $[Ca^{2+}]_i$, AA release by $cPLA_2$ and do not lead to 5-LO translocation. However, if combined with "priming" agents such as granulocyte macrophage colony-stimulating factor, tumor necrosis factor (TNF) α, phorbol esters, Epstein Barr virus or lipopolysaccharides (LPS), LTs are synthetized due to the activation of $cPLA_2$ (reviewed in (Werz, 2002)). Besides, those chemotactic agents also phagocytic particles like urate crystals (Serhan et al., 1984a,b) or opsonized zymosan (Claesson et al., 1981) lead to LT formation. Induction of cell-stress by e.g. osmotic shock, oxidative or chemical stress, leads to phosphorylation of 5-LO and subsequent LT synthesis (Werz et al., 2001a). By combining different stimuli with unique stimulation cascades, inside is gained into the molecular mechanisms of inhibitor actions.

Physiological and pathophysiological actions of LTs via distinct receptors

LTB_4 was described as a chemotactic agent (Ford-Hutchinson et al., 1980) and inducer of adhesion and rolling of leukocytes (Dahlen et al., 1981). LTB_4 recruits neutrophils from distant sites in the early phase of inflammation in murine models of sterile injury and infection (Lammermann et al., 2013) and it is suggested that LTB_4 enhances the fMLP-induced recruitment of neutrophils to inflammatory sites (Afonso et al., 2012). Additionally, LTB_4 stimulates the release of granules and superoxide anions from neutrophils (Hafstrom et al., 1981). B-lymphocytes are activated by LTB_4 and IL-4 induced secretion of immune globulins (IgM, IgG, IgE) is enhanced (Yamaoka et al., 1989, 1994). CysLTs cause vasoconstriction, bronchoconstriction and increase vascular permeability as well as plasma exudation (Dahlen et al., 1981; Drazen et al., 1980). LTs bind to specific GPCR. While LTB_4 targets BLT_1 and BLT_2, cysLTs unfold their action via $cysLT_1$ and $cysLT_2$ receptors (Yokomizo, 2011). Additionally, LTB_4 acts via PPARα and thereby interacts with the lipid catabolism that controls the duration of inflammation (Devchand et al., 1996). BLT_1 is the high-affinity receptor for LTB_4 and BLT_2, a low-affinity receptor (Yokomizo et al., 1997, 2000). Both share 45.2% identity in the amino acid sequence and show distinct differences in their responds towards pharmacological treatment (Yokomizo et al., 2000). For BLT_2, 12-HHT was identified as high-affinity ligand (Okuno et al., 2008) and 12-H(p)ETE and 15-HETE are also known to activate BLT_2 (Yokomizo et al., 2001).
While BLT_2 is ubiquitously expressed in humans, expression of BLT_1 is mainly concentrated on leukocytes (Yokomizo et al., 1997, 2000). BLT_1 is expressed on granulocytes, eosinophils, macrophages, bone marrow derived dendritic cells, osteoclasts and differentiated T-cells (Th1, Th2, effector CD8+ T cells) but not on naïve T-cells as reviewed by (Yokomizo, 2011). It is discussed to which degree BLT_1 is involved in the recruitment of immune cells to inflamed tissues in bronchial asthma as well as in multiple sclerosis, a disease governed by Th17 cells (Kihara et al., 2010; Miyahara et al., 2005; Terawaki et al., 2005). BLT_1 is one element of early recruitment of granulocytes into airways (Medoff et al., 2006). In mice models of inflammatory and collagen-induced arthritis, the BLT_1 receptor takes part in the recruitment of neutrophils (Chou et al., 2010; Kim et al., 2006; Shao et al., 2006). Studies with BLT_1 and apolipoprotein-E deficient mice revealed involvement of BLT_1 in the progression of cardiovascular diseases (Subbarao et al., 2004). In summary, involvement of LTB_4/BLT_1 pathway was described for atherosclerosis, aortic abdominal aneurysm, cerebrovascular diseases, multiple sclerosis, arthritis, pulmonary inflammation and

cancer (Back et al., 2011). Interestingly, knock-out of 5-LO does not lead to the same effects as BLT_1 knock-out in animal models of arteriosclerosis and experimental autoimmune encephalomyelitis since pro- as well as anti-inflammatory modulators are suppressed by knockout of 5-LO (Back et al., 2011).
CysLT receptors belong to the rhodopsin family and possess 7 transmembrane helices (Back et al., 2011; Sarau et al., 1999). Up to now two subtypes ($CysLT_1$ and $CysLT_2$) were cloned and further splicing variants of both types are discussed (Back et al., 2011). $CysLT_1$ and $CysLT_2$ share only 38% identity in their amino acid sequences and react differently towards classical CysLT receptor antagonists (montelukast, zafirlukast, pranlukast, pobilukast, MK571) (Back et al., 2011; Heise et al., 2000; Nothacker et al., 2000; Takasaki et al., 2000). $CysLT_1$ was cloned in 1999 and its expression shown in peripheral blood leukocytes (especially eosinophils), nasal mucosa, spleen, gastrointestinal system, reproductive system, alveolar macrophages and lung smooth muscle cells (Figueroa et al., 2001; Lynch et al., 1999; Sarau et al., 1999; Shirasaki et al., 2002). $CysLT_2$ is expressed in spleen, heart, adrenals, the reproductive system, peripheral blood leukocytes (especially eosinophils), lung and to a certain amount in the brain (Heise et al., 2000; Nothacker et al., 2000). Both receptors show unique responses towards agonists. For $CysLT_1$ receptor the binding affinity is $LTD_4 \gg LTC_4 > LTE_4$ and the potency at the $CysLT_2$ receptor is $LTD_4 = LTC_4 \gg LTE_4$ with LTE_4 acting as a partial agonist (Heise et al., 2000; Lynch et al., 1999; Nothacker et al., 2000; Sarau et al., 1999).
Interestingly, the targets of LTB_4 and cysLTs are clearly separated with LTB_4 predominantly acting on immune cells and cysLTs targeting smooth muscle cells and blood vessel cells (Samuelsson et al., 1987).

1.2.3 Cyclooxygenases and microsomal prostaglandin E_2 synthases

Besides LTs, also prostanoids derive from AA with prostaglandin (PG) E_2 as the most important metabolite (Fig. 1.10) (Samuelsson et al., 2007).

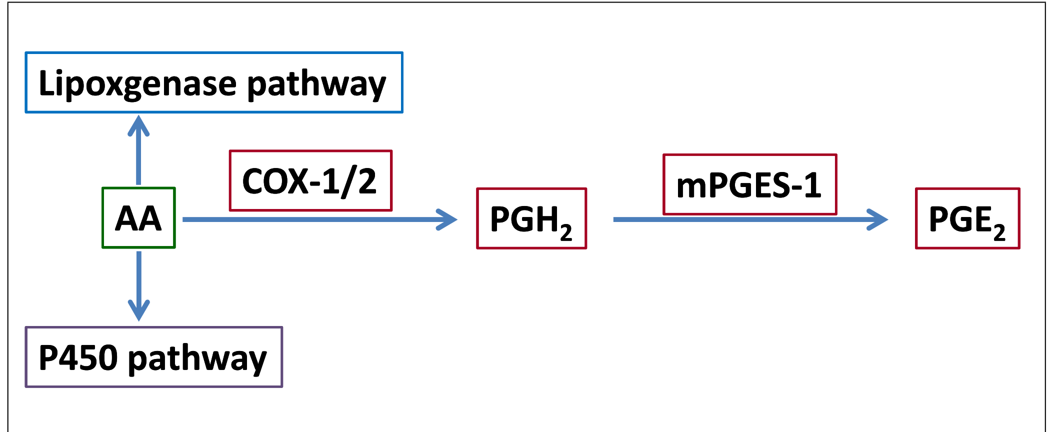

Figure 1.10: Overview of the PGE_2 formation pathway. COX = cyclooxygenase, PG = prostaglandin, mPGES-1 = microsomal prostaglandin synthase-1.

Membrane-bound COX enzymes catalyze the formation of instable intermediate PGG_2 and its corresponding alcohol PGH_2. So far, two isoforms (60% sequence identity) were identified – the constitutive COX-1 and the inducible COX-2 (Simmons et al., 2004). Both isoforms share catalytic features, but structural differences are known that led to the introduction of COX-2 selective drugs (Kurumbail et al.,

1996). From PGH_2, prostaglandins (PGD_2, PGE_2, $PGF_{2\alpha}$), prostacyclins (PGI_2) and thromboxanes (TXA_2) are quickly formed in the cellular environment by distinct synthases which catalyze non-oxidative rearrangements with exception of the generation of $PGF_{2\alpha}$. Up to now three PGE synthases were cloned and characterized: inducible microsomal prostaglandin E synthase-1 (mPGES-1) (Jakobsson et al., 1999), microsomal prostaglandin E synthase-2 (mPGES-2) (Tanikawa et al., 2002) and cytosolic prostaglandin E synthase (cPGES) (Tanioka et al., 2000). Additionally, soluble glutathione transferases catalyze the same reaction but the biological significance is still unclear (Smith et al., 2011). While mPGES-1 is involved in many inflammatory diseases, cPGES and mPGES-2 are connected to homeostasis and are ubiquitously expressed (Murakami et al., 2003; Smith et al., 2011). As LTC_4S and FLAP, mPGES-1 belongs to the MAPEG family and is GSH-dependent. In the cell, mPGES-1 seems to prefer COX-2 over COX-1 (Murakami et al., 2000). In the early phase of PGE_2 synthesis, cPGES is coupled to COX-1 (Tanioka et al., 2000) and mPGES-2 interacts with both COX-1 and 2 (Murakami et al., 2003). A structure of mPGES-1 from 2D crystals in presence of GSH (3.5 Å resolution) elucidated specific amino acids that are involved in the catalytic mechanism as well as the mode of GSH binding (Jegerschold et al., 2008). As many MAPEG proteins mPGES-1 forms homotrimers and incorporates three GSH molecules per homotrimer (Jegerschold et al., 2008; Thoren et al., 2003). Each monomer has four TM α-helices (Jegerschold et al., 2008). The catalytic reaction is not completely resolved. However, a glutathione peroxidase-like fashion was proposed. The reaction starts with the attack of the GSH thiol at the O-9 of PGH_2 and the formation of a sulfenate ester. A polarized H_2O withdraws the hydrogen at C-9 and, after breaking the ester bond at O-9, a ketone is formed (Smith et al., 2011). mPGES-1 expression is upregulated in various cells after stimulation with pro-inflammatory stimuli (e.g. LPS, TNFα) which is inhibited by glucocorticoids (Murakami et al., 2000; Thoren and Jakobsson, 2000; Uematsu et al., 2002). IL-1β induced mPGES-1 expression parallels with COX-2 expression in A549 cells (Jakobsson et al., 1999). PGE_2 mediates its various actions via GPCRs (EP1-EP4) (Kobayashi and Narumiya, 2002). The other prostanoids act through specific receptors, too: PGD_2 via DP1 and DP2 receptor; $PGF_{2\alpha}$ via FP receptor; PGI_2 via IP receptor and TXA_2 via TP receptor (Smith et al., 2011). PGE_2 is involved in fever (Engblom et al., 2003), inflammatory pain, collagen-induced arthritis in mice and swelling (Trebino et al., 2003). mPGES-1 is associated with rheumatic diseases and its expression was evident in synovial tissues from rheumatoid arthritis patients (Korotkova and Jakobsson, 2010; Westman et al., 2004). Increased expression of mPGES-1 was also shown in human cancer (e.g. colorectal cancer, breast cancer, lung cancer) (Mehrotra et al., 2006; Yoshimatsu et al., 2001a,b). mPGES-1 plays a critical role in stroke and related injury (Ikeda-Matsuo et al., 2006). Furthermore, mPGES-1 is upregulated in plaques from patients with carotid atherosclerosis. This suggests also a role in arteriosclerosis (Cipollone et al., 2004, 2001; Gomez-Hernandez et al., 2006).

1.3 The immune system during pregnancy

1.3.1 Regulation of the immune system during pregnancy

Pregnancy is accompanied by major changes of the maternal immune system in order to avoid the rejection of the semi-allogeneic fetus while protecting from pathogens

(Veenstra van Nieuwenhoven et al., 2003). Originally in 1953 Medawar hypothesized an overall suppression of the immune system during pregnancy or a lack of antigen presentation by the fetus (Sacks et al., 1999). However, further research revealed a complex network of up- and down regulation of the innate as well as the adaptive arm of the immune system (Fig. 1.11A).

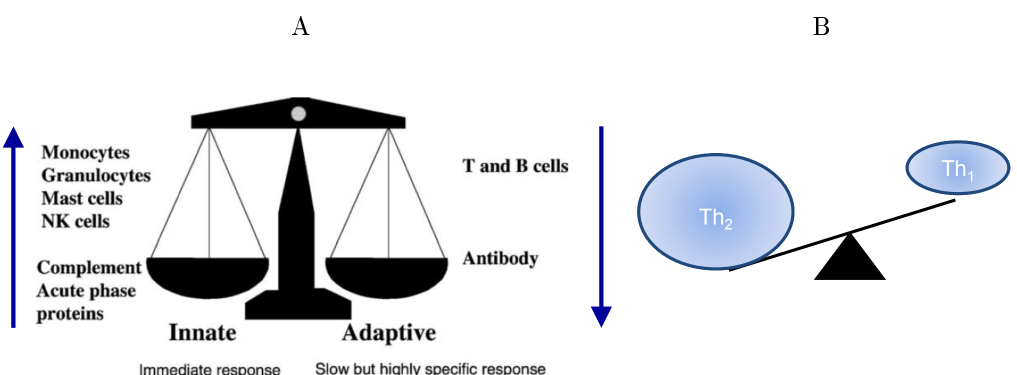

Figure 1.11: Immune system during pregnancy. (A) Regulation of the innative and adaptive arm of the immune system (modulated from (Luppi, 2003)). (B) The T helper cell 1 (Th_1) : T helper cell 2 (Th_2) paradigm.

One component of the adaptive immune system is the unique T helper cell 1 (Th1):T helper cell 2 (Th2) paradigm (Wegmann et al., 1993) (Fig. 1.11B). This paradigm describes a shift from the cell-mediated (Th1) to the humoral (Th2) control of the immune system. This was supported by a decrease of immunity against certain pathogens which are controlled by Th1 cells (Lysteria, Toxoplasma) and flares of related autoimmune diseases such as SLE which are mainly driven by Th2 cells during pregnancy (Wegmann et al., 1993). However, this paradigm is no longer completely accepted (Mor and Cardenas, 2010). The system is now enlarged towards the interaction between NK and trophoblast cells in the placenta in early pregnancy, which has further impact on the periphery (Sargent et al., 2006). Resent research favors a prominent role of specific regulatory T cells and reduced accumulation of effector T cells in the decidua due to the decreased expression of inflammatory chemokines (Aluvihare et al., 2004; Nancy et al., 2012). It was shown that fetal specific FOXP3+ regulatory T cells, which are suppressors of the immune system, are activated and accumulated. Additionally, memory T cells towards fetal antigens are formed which unfold their role in the next pregnancy (Rowe et al., 2012).

During pregnancy, the innate immune system in general seems to be upregulated. This is obvious by the elevation of granulocyte and monocyte numbers in pregnant females (Luppi et al., 2002b; Lurie et al., 2008; Minagawa et al., 1999). Furthermore, expression of certain pro-inflammatory surface markers on granulocytes and monocytes were increased during pregnancy and revealed similar activation patterns as in sepsis (Luppi et al., 2002a,b; Sacks et al., 1998). However, some responses are impaired. In *in vitro* experiments e.g., chemotaxis (Bjorksten et al., 1978), bacterial killing (Bjorksten et al., 1978), adherence (Krause et al., 1987) as well as ROS formation (Crouch et al., 1995; Kindzelskii et al., 2002, 2004) were reduced in maternal neutrophils. Dysregulation within this fine-tuned network leads to miscarriages or pre-eclampsia. The cause is usually a failing Th2 and predominant Th1 response as reviewed by (Sargent et al., 2006).

1.3.2 Influence of pregnancy on autoimmune disease activity

Certain autoimmune diseases such as rheumatoid arthritis (de Man et al., 2008) and multiple sclerosis (Confavreux et al., 1998) improve during pregnancy but relapses emerge after parturition. This is deduced from the role of regulatory T cells in the fetal-maternal tolerance, which might prevent autoimmune diseases (Munoz-Suano et al., 2012; Patas et al., 2013). Other diseases worsen such as SLE (Smyth et al., 2010) and asthma (Rey and Boulet, 2007). Especially during the second and third trimester, severe asthma tends to exacerbate (Rey and Boulet, 2007). There is a higher risk of fatal asthma exacerbations during pregnancy which might result from neutrophilia as well as higher Th2 cytokine ratio (Ali and Ulrik, 2013). Gestational rhinitis (prevalence: 20% of pregnant females) is distinct from allergic rhinitis and characterized by nasal congestion without allergic causes (Ellegard, 2003). In summary, LT related diseases are influenced by immunologic changes during pregnancy.

1.3.3 Involvement of LO products in pregnancy

LO derived products (LTs and HETEs) are formed in intrauterine tissues and 5-LO is expressed in fetal membranes (Brown et al., 1999; Jian et al., 2013; Mitchell and Grzyboski, 1987; Saeed and Mitchell, 1982). Involvement of LO products besides COX products was shown in the induction of delivery (Durn et al., 2010). 5-HETE for example induces the contractility of the human myometrium (Bennett et al., 1987). Recently it was found that LTB_4 enhances the innate immune defenses against the puerperal sepsis pathogen *Streptococcus pyogens*, which represents a leading cause of maternal mortality (Soares et al., 2013). At the peripheral level, it was shown that LTB_4 formation in granulocytes is reduced during pregnancy (Crocker et al., 1999; Imai and Arai, 1996).

1.3.4 Sex bias and LT formation

A sex bias was recently shown to be evident concerning the amount of LTs formed in human blood (Pergola et al., 2008). In blood, neutrophils and monocytes from female donors formed about twice as much LTs as compared to cells from male donors following stimulation with fMLP or A23187. In both cell types the suppression is caused by testosterone. In neutrophils, testosterone leads to activation of ERK and subsequent permanent localization of 5-LO at the nuclear membrane, where 5-LO seems to be catalytically less active (Pergola et al., 2008). In monocytes, ERK was also phosphorylated in response to testosterone leading to inactivation of PLD which further downregulates 5-LO activity (Pergola et al., 2011). The observed differences might partly explain the predisposition of females for certain autoimmune diseases such as SLE, asthma and allergic rhinitis (Whitacre, 2001).

1.4 Intervention of LT biosynthesis

The pharmacological intervention with LT synthesis takes place at different levels. Starting with the inhibition of overall substrate supply ($cPLA_2$), over direct interference of 5-LO product formation and downstream enzymes (LTA_4H, cysLT synthases) and ending with LT receptor antagonists (LTRA). All of these strategies were

tracked in order to find the best pharmacological treatment with minimal side effects. Interference with the 5-LO pathway is further subdivided into inhibitors that directly inhibit the enzyme or indirectly interact with 5-LO activation, FLAP or phosphorylation. So far only zileuton, a direct 5-LO inhibitor reached the market (Carter et al., 1991). From the other targets (BLT, LTA_4H, LTC_4S) only $CysLT_1$ receptor antagonists (montelukast, zafirlukast, pranlukast, pobilukast) are on the market for the treatment of asthma (Back et al., 2011).

Direct 5-LO inhibitors are separated into four groups according to their mode of inhibition: (I) redox-type, (II) iron ligand, (III) nonredox-type and (IV) novel-type or diverse 5-LO inhibitors (Pergola and Werz, 2010).

(I) Redox-type inhibitors

Redox-type inhibitors reduce the iron in the catalytic center of 5-LO and uncouple the redox-cycle (Fig. 1.12) (Rouzer et al., 1991).

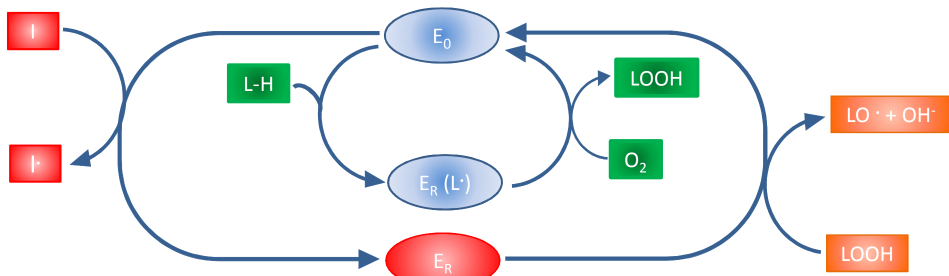

Figure 1.12: Scheme for the mechanism of redox-type inhibitors modified after (Rouzer et al., 1991). E_0 = oxidized, active 5-LO; E_r = reduced, inactive form of 5-LO; LOOH = fatty acid hydroperoxide; L-H = substrate; I = redox-type inhibitor. The inner cycle represents the catalysis of 5-LO and its substrate AA. The right part of the outer cycle illustrate the oxidative activation of 5-LO by lipid hydroperoxides. The left part shows the reductive inactivation of 5-LO by redox-type inhibitors.

These compounds are mainly lipophilic, own reducing properties and are often naturally-derived (e.g. flavonoids, coumarins, quinones, nordihydroguaiaretic acid, caffeic acid and polyphenols) (Werz, 2007). Examples of synthetic derivatives are AA-861, BW755C and L-656,224 (Fig. 1.13), which are highly efficient *in vitro*. Benzoquinones are potent inhibitors. They usually need bioactivation and are reduced to hydroquinones which then uncouple the redox cycle (Fig. 1.12) (Ohkawa et al., 1991b). Redox-type inhibitors are characterized by unspecific action since they target also other lipoxygenases' catalytic cycle. Further objectionable side effects are methemoglobin formation, radical species generation, low oral bioavailability and rapid metabolism (Ford-Hutchinson et al., 1994). The inhibitory efficiency does not correlate with redox potential, but rather with the lipophilicity of the compounds (Ford-Hutchinson et al., 1994). So far clinical studies with redox-type inhibitors were abandoned due to the poor compound characteristics.

Figure 1.13: Chemical structures of redox-type inhibitors.

(II) Iron ligand inhibitors

Iron ligand inhibitors (Fig. 1.14) are deduced from redox-type inhibitors. They show weak reductive properties and chelate the iron in the catalytic center (Corey et al., 1984). Most of the compounds from this group (like BWA4C) are highly potent *in vitro* (IC_{50} (BWA4C) = 40 nM, in cells) and some are also orally active (Tateson et al., 1988). However, clinically those hydroxamic acids were banned due to pharmacokinetic limits (extensive metabolism and metabolite accumulation) (Brooks and Summers, 1996; Tateson et al., 1988). Hydrolytic stable N-hydroxyurea derivatives were designed in order to lower the metabolic susceptibility and increase bioavailability. Zileuton (A-64077) turned out to be the most promising candidate in *in vitro* (IC_{50} = 0.5 – 1 μM in cells) as well as *in vivo* studies and was approved for asthma treatment in the USA (Zyflo®) (Carter et al., 1991; McGill and Busse, 1996). Up to now, it is still the only direct 5-LO inhibitor that reached the market. The immediate-release formulation was withdrawn in 2008 while the extended-release formulation is still approved. Atreleuton (VIA-2291, ABT-761) is a structurally optimized derivate of zileuton and shows higher potency and improved pharmacokinetics. Atreleuton reduced LT levels in urine as well as in stimulated blood and improved lung function in exercised-induced bronchoconstriction in asthma patients (Lehnigk et al., 1998). In a study (phase II trial, double-blind, placebo-controlled) in patients with recent acute coronary syndrome, atreleuton lowered LT levels in stimulated blood as well as in urine. The effect on artherotic plaques needs further studies to assure its benefits in the treatment of cardiovascular diseases (Tardif et al., 2010).

Figure 1.14: Chemical structures of iron ligand inhibitors.

(III) Nonredox-type inhibitors

Nonredox-type inhibitors (Fig. 1.15) are characterized by their competition with AA or lipid hydroperoxides and their inability to reduce or to chelate the catalytic iron

of 5-LO (Falgueyret et al., 1993). Due to the high variability of chemical structures, a clear 5-LO binding site was not identified yet (Pergola and Werz, 2010). This site could be either at the substrate-binding moiety or in an allosteric position. Importantly, though nonredox-type inhibitors do not interact with the catalytic iron by redox or ligand binding interaction, their potency is influenced by the overall redox tone in the cell (Werz and Steinhilber, 2005a). Attempts to design potent inhibitors devoid of redox properties successfully led to selective and orally active (methoxyalkyl)thiazoles and methoxytetrahydropyrans (Bird et al., 1991; Crawley et al., 1992; McMillan et al., 1991). A drawback is the observation that nonredox-type inhibitors lose their efficacy in presence of elevated peroxide levels and activation of 5-LO via phosphorylation, conditions that occur at inflammatory sides (Werz and Steinhilber, 2005b). Discrepancy between the inhibitory potential in cell-free 5-LO assays and intact cell assays is another feature of 5-LO inhibition (Fischer et al., 2004; Werz et al., 1998). The up to 100-fold lower inhibitory capacity in homogenates can be restored by addition of GSH or DTT in mM ranges, which activates the glutathione peroxidase activity and lowers the hydroperoxide tone (Werz et al., 1998). For some inhibitors of this class (namely ZM 230487, ZD 2138 or L-739,010) the mode of inhibition is switched in presence of peroxides from non-competitive to competitive binding (Werz et al., 1998). Compared to Ca^{2+}-mobilizing stimulation, ZM 230487 and L-739,010 lose potency following stimulation of cells via the kinase pathway (Fischer et al., 2003). However, CJ-13,610 inhibited equally well under both conditions (Fischer et al., 2004). PF-04191834 is deduced from CJ-13,610 and has improved pharmacological characteristics (Masferrer et al., 2010). Many nonredox-type inhibitors are orally-active, however devoid of efficacy in *in vivo* models. The urea derivative RBx 7796 is decorated with a dodecyl chain and was characterized as a competitive inhibitor not effected by the redox tone (Shirumalla et al., 2006). *In vivo* efficiency of this orally active compound was indicated in inflammatory models as well as bronchoconstriction (Shirumalla et al., 2006, 2008).

ZD-2138; R = CH_3
ZM-230487; R = CH_2-CH_3

CJ-13,610

L-739,010

RBx 7796

Figure 1.15: Chemical structures of nonredox-type inhibitors.

(IV) Novel-type inhibitors

Some inhibitors do not fit in either of the classes mentioned before. Examples are hyperforin from *Hypericum perforatum*, 3-O-acetyl-11-keto-boswellic acid (AKBA), licofelone (ML-3000) and sulindac sulfide (Fig. 1.16). Hyperforin seems to bind to the C2-like domain of 5-LO and thereby prevents the interaction with CLP and subsequent translocation. It is remarkably effective in *in vivo* models of carrageenan-induced pleurisy, which might origin in the dual inhibition of 5-LO and COX-1 (Albert et al., 2002; Feisst et al., 2009). AKBA selectively inhibits 5-LO most likely by Ca^{2+} dependent binding to a second regulatory AA-binding domain (Safayhi et al., 1992; Sailer et al., 1998). However, AKBA failed to inhibit LT formation in blood assays as well as in *in vivo* experiments (Siemoneit et al., 2009). Licofelone is a multiple 5-LO/COX-1/mPGES-1 inhibitor which is now tested in a phase III trial for the treatment of osteoarthritis (Alvaro-Gracia, 2004; Koeberle et al., 2008a; Laufer et al., 1994; Raynauld et al., 2009). Investigations of the molecular mechanism suggest that licofelone interferes with FLAP since it is a weak inhibitor of the purified 5-LO enzyme while potently reducing LT formation in cells. Additionally, translocation of 5-LO in cells was hindered by licofelone (Fischer et al., 2007). Sulindac was described as a non-selective COX-inhibitor. Its active metabolite sulindac sulfide directly inhibits 5-LO and intervenes with 5-LO translocation in an additive manner with MK-886 (Steinbrink et al., 2010).

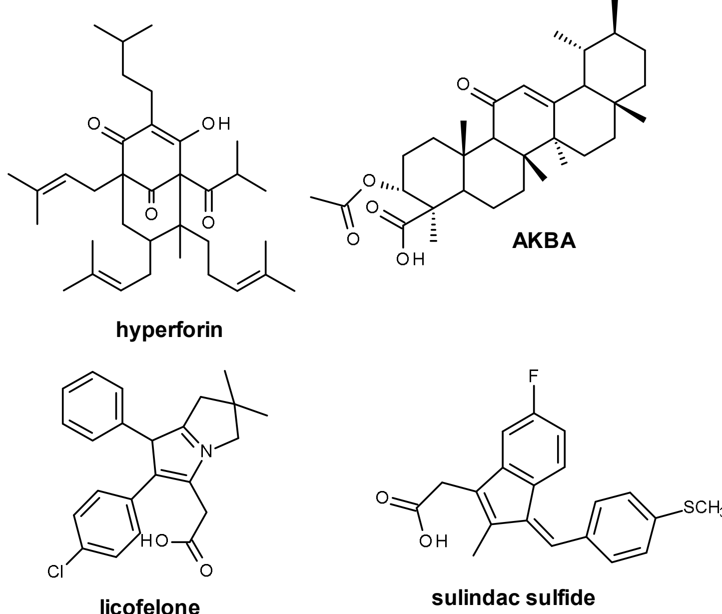

Figure 1.16: Chemical structures of diverse 5-LO inhibitors.

FLAP-antagonists

Another promising option to interfere with LT synthesis is the inhibition of FLAP and thereby the transfer of AA to 5-LO. FLAP antagonists show potent inhibition also in human *in vivo* studies. The first characterized compounds are the indole MK-886, the quinolone-indole MK-591 and the quinolone BAY X-1005 (Dahlen et al., 1997; Diamant et al., 1995; Friedman et al., 1993) (Fig. 1.17). But none of the above reached the market though benefits in allergic asthma and animal models for CVD were indicated (Evans et al., 2008). Interaction of MK-591 with FLAP was elucidated

by the crystal structure of the inhibitor-FLAP complex (Ferguson et al., 2007). Present intensive evaluation of FLAP inhibitors is conducted especially for AM-103 and its successor GSK2190915 (AM-803) (Hutchinson et al., 2009; Stock et al., 2011). In recent clinical studies GSK2190915 was regarded as safe and pharmacokinetics supported dosing once a day (Bain et al., 2013). Next, GSK2190915 was investigated in phase II trials for allergen induced asthma, in which only male subjects were included (Kent et al., 2013), and persistent asthma, where it was not superior over CysLT antagonist montelukast (Follows et al., 2013). Interestingly, in the second study only females were included due to observed testicular toxicity (Follows et al., 2013).

MK-886

MK-591

BAY X 1005

AM-103

GSK 2190915

Figure 1.17: Chemical structures of FLAP inhibitors.

1.4.1 Inhibitors of mPGES-1

Compounds that interfere of the formation of PGE_2 act either via the inhibition of mPGES-1 activity or by down-regulation of the mPGES-1 expression (e.g. by the benzothiophene γ-hydroxybutenolides (BTH) (Guerrero et al., 2007)). Myrtucommulone, garcinol, arzanol, curcumin, β-boswellic acid and epigallocathechin gallate are plant-derived inhibitors of mPGES-1 activity with IC_{50} ranging from 0.3 to 10 μM (Koeberle et al., 2009d,c; Bauer et al., 2011; Koeberle et al., 2009a; Siemoneit et al., 2011; Koeberle et al., 2009b). The first synthetic derivatives were derived from the 5-LO inhibitor MK-886 (Riendeau et al., 2005). Recently, the phenanthrene imidazole MF63 from the Merck Frosst Center was introduced, a selective and orally active inhibitor with high potency (IC_{50}: 1 nM) (Cote et al., 2007). MK-886, licofelone as well as pirinixic acid and its derivatives are dual inhibitors of mPGES-

1 and 5-LO (Koeberle and Werz, 2009). Inhibitor research was limited due to the fact that enzymes in humans, mice and rats structurally differ and discovered drug candidates often failed in animal models (Pawelzik et al., 2010; Xu et al., 2008). Additionally, shunting of the substrate PGH_2 towards other synthases, for example PGI_2 synthase, counteracts the benefits by mPGES-1 inhibition (Scholich and Geisslinger, 2006).

Figure 1.18: Chemical structures of mPES-1 inhibitors.

General aspects on pharmacological intervention of eicosanoid pathways

Traditionally, inflammatory diseases are treated with unselective NSAR, which are criticized for their severe side effects. Efforts to reduce renal and gastrointestinal adverse reactions, which might originate in suppression of PGE_2 and PGI_2 formation by unselective COX inhibition, with so-called Coxibs (COX-2 specific inhibitors) were not prosperous. Coxibs lead to increased risks of cardiovascular complications due to an imbalance of anti-thrombotic PGI_2 and pro-thrombotic TXA_2. Therefore minimizing the targets to single enzymes by inhibiting mPGES-1, the down-stream enzyme of COX-2, was a new strategy avoiding undesirable effects (Celotti and Laufer, 2001). However, single-target inhibitors also lead to side effects as shown for the coxibes (e.g. rofecoxibe, Vioxx by Merck). One problem is the shunting of liberated AA to other enzymes of the network since the activation route for $cPLA_2$ is the same for COX and LO enzymes (Hudson et al., 1993; Scholich and Geisslinger, 2006). Therefore, multiple-target inhibitors are now under construction to eliminate unwanted side-effects which often lead to withdrawal or hamper market launch of compounds (for review see also (Meirer et al., 2013). There are several options for combinations: 5-LO/COX-2, 5-LO/mPGES-1, COX-2/LTA_4H, and PLA_2/LTA_4H (He et al., 2012). Examples are licofelone targeting three enzymes/proteins (FLAP/mPGES-1/COX-1) (Fischer et al., 2007; Koeberle et al., 2008a), MK-886 (FLAP/mPGES-1) (Fischer et al., 2007; Riendeau et al., 2005), pirinixic acid derivaties (5-LO/mPGES-1) (Hieke et al., 2011; Koeberle et al., 2008b), celecoxib (5-LO/COX-2) (Maier et al., 2008) and classical NSARs modified for dual inhibition of 5-LO and mPGES-1 (Elkady et al., 2012).

2 Aim of this study

LTs are fast reacting pro-inflammatory mediators of the immune system (Afonso et al., 2012; Muller et al., 2009). Besides their physiological roles, they are involved in the onset of inflammatory diseases such as asthma, allergic rhinitis, SLE, rheumatoid arthritis and also cardiovascular diseases and cancer (Haeggström et al., 2010). The treatment of LT related diseases by specific inhibitors is regarded as an alternative to the traditionally used routes. So far various direct or indirect inhibitors of 5-LO product synthesis and receptor antagonists were designed and their potential assessed (Back et al., 2011; Pergola and Werz, 2010). In this respect, it is important to understand the physiological aspects of inflammation and LT biosynthesis. During the last years, growing attention was directed to the differences of the male and female immune system and relating consequences in the individual pharmacotherapy (Whitacre, 2001). With regards to the sex bias observed for LT biosynthesis in male and female derived blood the question arose if and how pregnancy influences LT synthesis (Pergola et al., 2008, 2011). Pregnancy is a unique state in the life of women which is accompanied by high fluctuations of hormones. Interestingly, during pregnancy the maternal immune system undergoes tremendous changes in order to tolerate the fetal allograft (Veenstra van Nieuwenhoven et al., 2003). In the same manner the course of certain immune diseases changes during pregnancy (e.g. rheumatoid arthritis, multiple sclerosis, SLE, asthma, rhinitis) (Confavreux et al., 1998; de Man et al., 2008; Rey and Boulet, 2007; Smyth et al., 2010). LTRA are used as treatment of asthma during pregnancy (Schatz and Dombrowski, 2009). However, this application is not supported by clinical studies and the use is extrapolated from studies with non-pregnant female patients. It was shown that LTs play a role in implantation and at term in a similar way as prostanoids (Durn et al., 2010). Fetal and maternal tissues express LOs and LO products were detected (Brown et al., 1999; Jian et al., 2013; Mitchell and Grzyboski, 1987; Saeed and Mitchell, 1982). Furthermore, LT formation is reduced in isolated peripheral cells from pregnant donors and in serum lower levels of LTB_4 were registered during the second trimester (Crocker et al., 1999; Jian et al., 2013). Up to now it has not been studied in detail if and how pregnancy impacts peripheral LT biosynthesis and if so, which consequences would result for the physiological role of LTs during pregnancy. Therefore, the aim of this thesis was to investigate if pregnancy influences LT formation and if so, to elucidate the underlying mechanisms. In order to investigate this, blood assays in which the cells are in their physiologic environment as well as isolated cells and plasma preparations were used. Besides the importance to learn more about the physiological and pathophysiological regulation of mediators in the immune system, pharmacological intervention is always studied side by side. Targeting 5-LO with specific inhibitors to treat LT related diseases attacks LT formation as early as the first catalytic step without affecting other AA derived routes. However, so far attempts failed due to various reasons. The only drugs that reached the market are zileuton as direct 5-LO inhibitor and CysLT antagonists for the treatment of asthma (Back et al., 2011; Carter et al., 1991). Although many inhibitors of various types of chemical structu-

res are published, detailed analysis of the molecular interactions with 5-LO are rare. Special focus was therefore placed on the mode of action of benzoquinones and their molecular pharmacological characterization. It is known that naturally occurring benzoquinones interfere with LT formation (Werz, 2007). However, the inhibitory mechanism was not identified. In this thesis, embelin was identified as 5-LO and mPGES-1 inhibitor and underlying molecular mechanisms were elucidated. Molecular docking studies by the group of Dr. Daniela Schuster, University of Innsbruck, Austria were used to review the biological results. Traditionally, benzoquinone-type 5-LO inhibitors are grouped as redox-type or ligand-type inhibitors. Using the example of embelin as well as the related benzoquinone RF-Id, this classification was critically re-evaluated. Next, structure activity relationships (SAR) of newly designed benzoquinone structures were investigated which were supplied by the group of Dr. Rosanna Filosa, University of Salerno, Italy. These structures are composed of benzoquinone cores connected to lipophilic alkyl chains and thereby resemble fatty acids. Fatty acids is known to modulate cellular LT biosynthesis (Iversen et al., 1991; Peters-Golden and Shelly, 1988; Vasange-Tuominen et al., 1994). Interestingly, n-hexyl or n-octyl chains in α-position of pirinixic acid derivatives increase their inhibitory potential (Koeberle et al., 2008b; Werz et al., 2008). Besides SAR studies, selectivity for 5-LO inhibition in respect to related enzymes as well as consequences of structural variations regarding potency in blood experiments, were part of the investigations. Biological characterization of the most potent compounds was done in respect to the mechanism of inhibition, selectivity, potency in cell-free and cellular test systems and in blood.

3 Materials and Methods

3.1 Materials

[5,6,8,9,11,12,14,15-^{3}H]-AA	Biotrend, Cologne, Germany
11β-PGE_2	Cayman Chemical, Ann Arbor, USA
5α-DHT	Sigma-Aldrich, Deisenhofen, Germany
AA-861	Sigma-Aldrich, Deisenhofen, Germany
adenosine deaminase	Calbiochem/Merck KGaA, Darmstadt, Germany
ampicillin	AppliChem, Darmstadt, Germany
arachidonic acid	Sigma-Aldrich, Diesenhofen, Germany
ATP	Roche diagnostics, Mannheim, Germany
BCIP	AppliChem, Darmstadt, Germany
BPB	Merck, Darmstadt, Germany
BSA, essentially fatty acid free	Sigma-Aldrich, Deisenhofen, Germany
Ca^{2+}-ionophore A23187	Sigma-Aldrich, Deisenhofen, Germany
$CaCl_2$	AppliChem, Darmstadt, Germany
celecoxib	Sigma-Aldrich, Deisenhofen, Germany
chloroform	Sigma-Aldrich, Deisenhofen, Germany
citric acid, monohydrate	AppliChem, Darmstadt, Germany
COX-1, ovine	Cayman Chemical, Ann Arbor, MI, USA
COX-2, human	Cayman Chemical, Ann Arbor, MI, USA
DCF-DA	Sigma-Aldrich, Deisenhofen, Germany
dextran-coated charcol	Sigma-Aldrich, Deisenhofen, Germany
dextrane	Sigma-Aldrich, Deisenhofen, Germany
DMEM/High glucose (4.5 g/l)/stable L-glutamine	PAA, Coelbe, Germany
DMSO	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
D-PBS	Serva Electrophorsis, Heidelberg, Germany
DPI	Sigma-Aldrich, Deisenhofen, Germany
DPPH	Cayman Chemical, Ann Arbor, MI, USA
DTT	AppliChem, Darmstadt, Germany
EDC	Calbiochem/Merck KGaA, Darmstadt, Germany
EDTA	AppliChem, Darmstadt, Germany
estradiol	Sigma-Aldrich, Deisenhofen, Germany
FCS	Sigma-Aldrich, Deisenhofen, Germany
$FeCl_2$	AppliChem, Darmstadt, Germany
$FeSO_4$ 7$\times H_2O$	AppliChem, Darmstadt, Germany
fMLP	Sigma-Aldrich, Diesenhofen, Germany
Fura-2/AM	Sigma-Aldrich, Deisenhofen, Germany
glucose	AppliChem, Darmstadt, Germany
glycerol	Caesar & Loretz GmbH, Hilden, Germany
glycine	AppliChem, Darmstadt, Germany
GSH, reduced	AppliChem, Darmstadt, Germany

hemoglobin	Sigma-Aldrich, Deisenhofen, Germany
IL-1β	ReproTech, Hamburg, Germany
imidazol	Sigma-Aldrich, Deisenhofen, Germany
indometacin	Sigma-Aldrich, Deisenhofen, Germany
Insect Express SF9-S2 medium	PAA, Coelbe, Germany
ionomycin	Sigma-Aldrich, Deisenhofen, Germany
IPTG	AppliChem, Darmstadt, Germany
leupeptin	Sigma-Aldrich, Deisenhofen, Germany
L-glutamine	PAA, Coelbe, Germany
LPS from *E.coli* 0127 B8	Sigma-Aldrich, Diesenhofen, Germany
LSM 1077	PAA, Coelbe, Germany
lysozyme	Sigma-Aldrich, Deisenhofen, Germany
$MgSO_4$ 7×H_2O	AppliChem, Darmstadt, Germany
MK-886	generous gift from Dr. L. G. Garland (Wellcome Res. Laboratories)
MTT	Sigma-Aldrich, Deisenhofen, Germany
NaCl	AppliChem, Darmstadt, Germany
NBT	Roche Diagnostics, Mannheim, Germany
Nonidet P-40	Applichem, Darmstadt, Germany
p-anisidinium chloride	Calbiochem/Merck KGaA, Darmstadt, Germany
PAPC	Avanti Polar Lipids Inc., Alabaster, AL, USA
penicillin/streptomycin solution	PAA, Coelbe, Germany
peptone	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
PGB_1	Sigma-Aldrich, Diesenhofen, Germany
PGH_2	Larodan, Malmö, Sweden
PMA	Applichem, Darmstadt, Germany
PMSF	Sigma-Aldrich, Germany
POG	Avanti Polar Lipids Inc., Alabaster, AL, USA
ponceau	Sigma-Aldrich, Deisenhofen, Germany
progesterone	Sigma-Aldrich, Deisenhofen, Germany
RPMI 1640	PAA, Coelbe, Germany
SDS	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
STI	Sigma-Aldrich, Deisenhofen, Germany
sucrose	AppliChem, Darmstadt, Germany
TEA	Sigma-Aldrich, Deisenhofen, Germany
TFA	AppliChem, Darmstadt, Germany
trypanblue	Sigma-Aldrich, Deisenhofen, Germany
trypsin-EDTA	PAA, Coelbe, Germany
Tween-20®	Carl Roth GmbH & Co. KG Germany
yeast extract	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
zileuton	Sequoia Research Products (Oxford, UK)
β-mercaptoethanol	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
γ-linolenic acid	Cayman Chemical, Ann Arbor, MI, USA

Compounds

Embelin, embelin-derived benzoquinones and RF- Id were synthesized by the group of Dr. Rosanna Filosa, University of Salerno, Italy. All stocks were prepared in DMSO and stored at $-20\,°C$.

3.2 Methods

3.2.1 Description of the study to investigate LT formation during pregnancy

Blood samples were pairwise collected from healthy pregnant and non-pregnant donors in Tuebingen (November 2008- July 2010; approval numbers: 338/2006V and 148/2010BO2) and Jena (December 2010-November 2011; approval number: 3197-07/11), with informed consent. All subjects had no apparent inflammatory conditions at the time of blood withdrawal and had not taken any anti-inflammatory drug for at least ten days prior to blood collection. Non-pregnant controls had not taken any hormones. Out of 30 pregnant donors, 27 were finally included in the study. The different pairs were analyzed in different experimental days except one case, when two pregnant women were analyzed at the same day and one non-pregnant donor was used as control. Additionally, two pregnant donors were analyzed twice (once during the second and once during the third trimester). In total, 3 pregnant women were excluded from the study for the following reasons: one donor experienced preeclampsia thereafter, one donor reported pregnancy rhinitis during the two previous pregnancies, and one pregnant female was excluded since the corresponding non-pregnant control had hyperandrogenism (high total serum testosterone levels, namely 5.3 nM) (Pergola et al., 2008). Withdrawn blood was immediately analyzed or used for experiments. Out of the 27 pairs, 5 pairs were analyzed by Dr. Carlo Pergola, University of Jena, Germany.

3.2.2 Quantification of blood cells and sex hormones

Peripheral venous blood (30 to 40 ml) was collected in heparinized tubes (16 I.E. heparin/ml blood, Sarstedt, Nuembrecht, Germany)) or EDTA tubes (1.6 mg EDTA-K_3/ml, Sarstedt, Nuembrecht, Germany)) by venipuncture. Total serum estradiol, progesterone and testosterone were analyzed by an automated chemiluminescence immunoassay systems (ADVIA Centaur, Siemens Medical Solution), according to the manufacturer's instructions. The complete blood count was performed by flow cytometry at the central laboratories of the University Hospital Tuebingen.

3.2.3 Isolation of plasma from human blood and stripping of the plasma

Human plasma was isolated from heparinized blood (16 I.E./ml) by centrifugation $600 \times g/10$ min/4 °C. The supernatant was centrifuged again ($800 \times g/10$ min/4 °C), and the resulting supernatant was analyzed to confirm the absence of cellular contaminations. To remove hormones plasma was stripped with 20 μg dextran-coated charcoal per ml plasma over night with continuous agitation at 4 °C according to manufacturer's instructions. After centrifugation twice at $2000 \times g/15$ min/ 4 °C the stripped plasma was sterile filtered (0.22 μm pore-size, Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Plasma was stored at −80 °C until use.

3.2.4 Cells

Isolation of human blood cells

Human primary cells (platelets, PMNL and monocytes) were isolated from leucocyte concentrates generated at the blood centers of the University Hospitals of Tuebingen and Jena. Cells used in the pregnancy study were isolated directly from heparinized blood after separation from plasma. Buffy coat cells were promptly isolated at 4 °C by dextran sedimentation (5% dextrane from Leuconostoc spp., (Mr = 500000 g/mol) (w/v) in dulbecco's phosphate buffer saline (D-PBS)) followed by density centrifugation on LSM 1077 (lymphocyte separation medium) cushions at 1,000 × g/10 min/4 °C without brakes. Platelets were isolated from platelet-rich plasma from the supernatants after density gradient centrifugation. Platelet-rich plasma was mixed with ice-cold D-PBS pH 5.9 (3:2) and centrifuged at 2,100 × g/15 min/4 °C. The resulting pellet was resuspended in a mixture of ice-cold D-PBS pH 5.9 and 0.9% NaCl (1:1) and centrifuged at 1,900 × g/10 min/4 °C. Platelets were resuspended in ice-cold D-PBS pH 7.4, and after addition of 1 mM $CaCl_2$ immediately used for experiments. After density centrifugation, PMNL were isolated from the pellets after hypotonic lysis of erythrocytes as described (Werz et al., 2002a). PMNL were resuspended in ice-cold D-PBS buffer containing 1 mg/ml glucose (PG) buffer. Monocytes were isolated from peripheral blood mononuclear cells (PBMC) which were collected after density gradient centrifugation as described (Pergola et al., 2011). For this, PBMC were resuspended in RPMI 1640 containing 10% fetal calf serum (FCS), 2 mM L-glutamine and penicillin (100 U/ml)/streptomycin (100 μg/ml) and with a cell density of 2×10^7/20 ml cells seeded to 175 cm^2 culture flasks (Greiner, Nuertingen, Germany). For assays where isolated monocytes were incubated in human plasma no serum was added to exclude hormone effects from the FCS. After 1.5 h at 37 °C, 6% CO_2 lymphocytes were removed by gentle shaking and two washing steps with D-PBS. Adherent monocytes were detached and resuspended in PG buffer. Directly before the assays 1 mM $CaCl_2$ was added. Cells were either mixed with trypanblue solution (1:1, 0.2%) and counted with a buerker haemocytometer under a light microscope or with a Vi-cell counter (Beckmann Coulter GmbH, Krefeld).

A549 cells

A549 cells (human lung epithelial carcinoma cell line) were obtained from Prof. Dr. Olof Rådmark (Karolinska Institute, Stockholm, Sweden). A549 cells were grown in DMEM/High glucose (4.5 g/l)/stable L-glutamine with 10% FCS and penicillin (100 U/ml)/streptomycin (100 μg/ml). Every third day cells were detached with trypsin-EDTA for 10 min and seeded at a concentration of 2×10^6/20 ml medium in 175 cm^2 culture flasks. Cells were grown in a Heracell incubator at 37 °C, 6% CO_2 (Heraeus, Hanau, Germany).

Sf9 cells

Sf9 cells (insect cells from Spodoptera frugiperda) were obtained from Prof. Dr. Jesper Z. Haeggström (Karolinska Institut, Stockholm, Sweden). Cells were grown in suspension culture at 27 °C continuously stirring in an incubator (Binder GmbH, Tuttlingen, Germany). Cells were seeded in Insect Express SF9-S2 medium supplemented with 10% FCS and penicillin (100 U/ml)/streptomycin (100 μg/ml) with a cell density of 5×10^5/ml and split at a density of 2×10^6/ml.

3.2.5 Determination of lipoxygenase products in human blood

For assays in human whole blood, freshly withdrawn blood from healthy adult donors was obtained by venipuncture and collected in monovettes containing 16 I.E./ml heparin (Sarstedt, Nuembrecht, Germany) at the Transfusion Center of the University Hospitals of Tuebingen and Jena. For the studies in blood from pregnant donors the experiments were conducted leaving out the preincubation steps for the inhibitors. Aliquots of 2 ml were pre-incubated with the test compounds or with vehicle (0.1% DMSO) for 15 min at 37 °C, as indicated, and formation of 5-LO products was started by addition of 30 μM Ca^{2+}-ionophore A23187 for 10 min at 37 °C. Alternatively, blood was first primed with 1 μg/ml lipopolysaccharide from E.coli 0127:B8 (LPS) for 15 min at 37 °C before the addition of compounds or vehicle (0.1% DMSO) for another 15 min at 37 °C and then stimulated with 1 μM fMLP for 15 min at 37 °C (Surette et al., 1993). The reaction was stopped on ice and the samples were centrifuged (600 × g/10 min/ 4 °C). Aliquots of the resulting plasma (500 μl) were then mixed with 2 ml of methanol and 200 ng prostaglandin B_1 (PGB_1) were added as internal standard. The samples were placed at −20 °C for 2 h and centrifuged again (600 × g/15 min/4 °C). The supernatants were collected and diluted with 2.5 ml D-PBS and 75 μl of 1 N HCl. Formed 5-LO metabolites were extracted and analysed by HPLC as described for intact cells (Pergola et al., 2008).

3.2.6 Determination of lipoxygenase products in cellular test systems

For the determination of lipoxygenase products from 5-LO, 12-LO and 15-LO in cells, PMNL or monocytes (1 to 5 × 10^6/ml) were resuspended in PGC buffer (D-PBS containing 1 mg/ml glucose and 1 mM $CaCl_2$). For inhibitor studies, cells were preincubated with compounds or vehicle (0.1% DMSO) for 15 min at 37 °C followed by induction of product formation with 2.5 μM Ca^{2+}-ionophore A23187 or 2.5 μM Ca^{2+}-ionophore A23187 plus 20 μM AA at 37 °C. After 10 min the reaction was terminated by the addition of 1 ml methanol and 30 μl 1 N HCl, 500 μl D-PBS pH 7.4 and 200 ng PGB_1. Alternatively, PMNL (5 × 10^6/ml PGC buffer) were first primed with 1 μg/ml LPS for 15 min at 37 °C before the addition of the compounds or vehicle (0.1% DMSO). After another 5 min, 0.3 U/ml adenosine deaminase was added and 10 min later, 5-LO product formation was started by the addition of 1 μM fMLP for 5 min at 37 °C (Flamand et al., 2000). The reaction was stopped on ice and the samples were centrifuged (800 × g/5 min/4 °C) and 800 μl of the supernatant added to 800 μl of methanol. After addition of 24 μl 1 N HCl, 400 μl D-PBS pH 7.4 and 200 ng PGB_1 samples were extracted. Formed metabolites were extracted with solid phase extraction prior to analysis on HPLC. For this, samples were centrifuged (800 × g/10 min/20 °C) and transferred to C_{18} solid phase columns (100 mg, UCT, Bristol, PA, USA) which were preconditioned with 1 ml methanol and 1 ml H_2O. After washing with 1 ml H_2O and 1 ml 25% methanol, metabolites were eluted with 300 μl methanol into 120 μl H_2O. 50 or 100 μl of the extracts were analyzed via HPLC on a C_{18} column (Nova-Pak cartridge, 5 × 100 mm, 4 μm particle size, Waters, Eschborn, Germany) eluted with methanol/H_2O/trifluoroacetic acid (TFA) (24/76/0.007) as mobile phase (Steinhilber et al., 1989). The amounts of the metabolites were quantified after peak area integration at the basis of PGB_1 as internal standard. 5-LO products analyzed by this method were LTB_4 and its

all-trans isomers, 5(S)-hydroxy-6-*trans*-8,11,14-cis-eicosatetraenoic acid (5-HETE), and 5(S)-hydroperoxy-6-*trans*-8,11,14-cis-eicosatetraenoic acid (5-H(p)ETE). LTC_4, D_4 and E_4 were below the detection limit and oxidation products of LTB_4 were not determined with this method. PGB_1, LTB_4 and its all-trans isomers were quantified at 280 nm and 5-HETE and 5-H(p)ETE as well as 12-HHT, 12-H(p)ETE and 15-H(p)ETE at 235 nm. Freshly isolated monocytes (5×10^6 cells) were resuspended in 1 ml PGC buffer, primed with 1 μg/ml LPS for 5 min at 37 °C, and incubated with the test compounds or vehicle (0.1% DMSO) for 15 min at 37 °C and stimulated with 1 μM fMLP for 10 min at 37 °C (Surette et al., 1996). The reaction was stopped on ice, and the supernatants were collected after centrifugation (600 × g/10 min/4 °C). LTC_4, D_4 and E_4 were determined in the supernatants with enzyme immunoassay (EIA) according to manufacturer's (Enzo Life Sciences International Inc., Lörrach, Germany) instructions. LTB_4 was analyzed by HPLC after solid phase extraction.

3.2.7 Determination of COX-1 product 12-HHT in platelets

Freshly isolated platelets (10^8/ml) were resuspended in PGC buffer. After 15 min of preincubation with compounds or vehicle (0.1% DMSO) at 37 °C samples were stimulated with 5 μM AA for 10 min at 37 °C. The reaction was stopped by the addition of methanol and the samples were treated as described for 5-LO product formation in cells. Formed COX-1 product 12-HHT was analyzed by HPLC (Albert et al., 2002).

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

E.coli (BL21) was transformed with pT3-5-LO plasmid, and recombinant 5-LO protein was expressed as described with minor modifications (Hammarberg et al., 1995). In brief, *E.coli* was cultured in LB-medium (5 g/l yeast extract, 10 g/l NaCl, 10 g/l peptone, pH 7.2) with 100 μg/ml ampicillin, 5 μM $FeSO_4$ and 2 mM $MgSO_4$ for 4-6 h at 30 °C until an OD_{620} of 0.2-0.3 was reached. Isopropyl-β-D-thiogalactopyranoside (190 μg/l) was added to induce the expression of the 5-LO protein over night at 30 °C. Cells were harvested by centrifugation at 7,700 × g for 15 min at 4 °C and resuspended in lysis buffer containing 50 mM triethanolamine (TEA)/HCl pH 8.0, 5 mM EDTA , 60 μg/ml soybean trypsin inhibitor (STI), 1 mM phenylmethanesulphonyl fluoride (PMSF), 2 mM DTT and lysozyme from chicken egg white (1 mg/ml). After 45 min lysis on ice, cells were sonicated for 3 × 15 s and centrifuged (40,000 × g/20 min/4 °C). To purify the 5-LO enzyme protein, the supernatant was applied to affinity chromatography using an ATP-agarose column (Sigma A2767, Sigma-Aldrich, Taufkirchen, Germany) which was equilibrated with D-PBS-EDTA (1 mM). After washing steps with 50 mM phosphate buffer pH 7.4 containing 1 mM EDTA and 0.5 M NaCl followed by 50 mM phosphate buffer pH 7.4 plus 1 mM EDTA, the 5-LO protein was eluted with extensive amounts of ATP (20 mM, in 50 mM phosphate buffer and 1 mM EDTA) (Brungs et al., 1995).

3.2.9 Generation of the 5-LO mutant

The Y181A mutant of 5-LO was generated via PCR mutagenesis by Jana Gerstmeier, University of Jena, Germany according to manufacturer's instructions (Quik-

Change Site directed mutagenesis kit (Stratagen, La Jolla, CA)). In brief, the PCR mixture (total volume 50 μl) contained out of reaction buffer, 250 μM dNTPs, 0.5 μM Fprimer (GTG GAC TTT GTT CTG AAT GCC TCC AAA GCG ATG GAG AAC CTG), 0.5 μM Rprimer (CAG GTT CTC CAT CGC TTT GGA GGC ATT CAG AAC AAA GTC CAC), 10 ng pT3 plasmid containing wild-type 5-LO, 3% DMSO and 0.05 U/μl Pfu Turbo DNA polymerase was applied to the PCR protocol described in Table 3.2. After digestion of the parental dsDNA with DpNI restriction enzyme, the DNA was transformed into XL1-Blue supercompetent cells to amplify the plasmid. Several colonies were picked, the plasmids isolated by Miniprep (GeneJET plasmid Miniprep, Thermo Scientific, Schwerte, Germany) and sequenced. The correct plasmid was transformed into competent TOP10 and BL21 *E.Coli* (work of Jana Gerstmeier). The 5-LO enzyme expressed as described (3.2.8).

Table 3.2: PCR protocol for PCR mutagenesis.

cycle step	temperature (°C)	time	cycles
initial denaturation	95	2 min	1
denaturation	95	30s	15
annealing	55	30s	
extension	72	11 min (2 min /kb)	
final extension	72	10 min	1
hold	4	hold	1

3.2.10 Determination of lipoxygenase products in cell-free systems

Partially purified 5-LO was diluted with D-PBS containing 1 mM EDTA and 1 mM ATP. 1 ml enzyme solution per sample was preincubated with compounds or vehicle (0.1% DMSO) for 15 min on ice. After prewarming for 30 s at 37 °C the product formation was induced by the addition of 2 mM $CaCl_2$ (final concentration 1 mM) and 20 μM AA. After 10 min at 37 °C, the reaction was terminated by the addition of 1 ml methanol. 500 μl D-PBS pH 7.4, 30 μl 1N HCl and 200 ng PGB_1 were added and the samples were treated as described for the 5-LO product formation in cells.

3.2.11 Generation of cell homogenates

For the generation of cell homogenates, PMNL or PBMC (5×10^7/ml) were preincubated in D-PBS containing 1 mM EDTA for 5 min at 4 °C. Cell disruption by sonication (3×10 s) at 4 °C was confirmed by light microscopy with trypanblue exclusion. The cell homogenate was diluted 10-fold to reach a corresponding cell concentration of 5×10^6/ml. After preincubation with compounds or vehicle (0.1% DMSO) for 15 min at 4 °C, 1 mM ATP was added and samples were prewarmed at 4 °C for 30 s. 5-LO product formation was induced by stimulation with 2 mM $CaCl_2$ (final concentration 1 mM $CaCl_2$) and 20 μM AA for 10 min at 37 °C. The reaction was terminated by addition of 1 ml methanol and the samples treated as described for isolated 5-LO enzyme.

3.2.12 Expression and purification of $cPLA_{2\alpha}$

The baculovirus, carrying the genetic information of the His-tagged $cPLA_{2\alpha}$ protein was kindly provided by Dr. M. Hoffmann (University of Frankfurt, Germany). For this, the $cPLA_{2\alpha}$ coding sequence was cloned from pVL1393 plasmid (kindly provided by Dr. Wonhwa Cho, University of Illinois at Chicago) into pFastBac™HTa containing a 6 x His-tag-coding sequence. The recombinant plasmid was transformed into DH10Bac™*E. coli.*. Sf9 cells were transfected with recombinant bacmid DNA using Cellfectin® Reagent and the generated baculovirus was amplified (Hoffmann et al., 2010). Sf9 cells were infected with the baculovirus for 72 h. Cells were harvested by centrifugation (600 × g/10 min/4 °C) and resuspended in lysis buffer containing 50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 10% glycerol (v/v), 1 mM EDTA, 60 μg/ml STI and 1 μg/ml leupeptin. After sonication (4 × 10 s), the cell debris was removed by centrifugation (100,000 × g/1 h/4 °C). The supernatant was collected and 2 mM $MgSO_4$ and 10 mM imidazole was added. The $cPLA_{2\alpha}$ protein was purified with affinity chromatography using Ni-NTA Agarose (Quiagen GmbH, Hilden, Germany). The agarose was equilibrated according to the manufacturer's instructions and the sample was incubated with the Ni-NTA suspension (100 μl Ni-NTA agarose/ml sample) by gently shaking for 1-2 h at 4 °C. Ni-NTA agarose was transferred to a column and washed 6 × with washing buffer (50 mM NaH_2PO_4 pH 8, 300 mM NaCl, 10% glycerol (v/v), 20 mM imidazole pH 8) 400 μl/1ml sample. The enzyme protein was eluted with elution buffer (50 mM NaH_2PO_4 pH 8, 300 mM NaCl, 10% glycerol (v/v), 150 mM imidazole pH 8) 300 μl/1 ml sample. Next, the enzyme solution was dialyzed to remove the imidazole against TGN buffer (10 mM trimethanolamine (Tris)/HCl pH 8, 20% glycerol (v/v), 300 mM NaCl). For dialysis, a regenerated cellulose dialysis membrane with a cutoff of 25,000 (Spectra/Por®, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) was used which has been boiled in saturated EDTA-solution before use. After 1-2 h, the TGN buffer was replaced by fresh buffer solution and dialysed over night at 4 °C. The dialysate was stored at −80 °C until use. The protein concentration was quantified with a modified Bradford assay (Roti®Nanoquant, Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Per sample 50 ng $cPLA_{2\alpha}$ protein was used.

3.2.13 Determination of arachidonic acid release from phospholipid vesicles

Multilamellar vesicles (MLVs) were prepared by drying PAPC and 1-palmitoyl-2-oleoyl-*sn*-glycerol (POG) in a ratio of 2:1 (nmol:nmol) in chloroform under nitrogen in glass vials. After addition of 20 mM Tris buffer (pH 7.4) containing 134 mM NaCl and 1 mg/ml bovine serum albumin (BSA), essentially fatty acid free (faf), the MLV suspension was disrupted by several freeze-thaw cycles (liquid nitrogen) and then extruded 11 times with a mini-extruder (Avanti Polar Lipids, Inc., Alabaster, AL, USA) through a polycarbonate membrane (100 nm pore diameter, Avanti Polar Lipids, Inc., Alabaster, AL, USA) at room temperature (above transition temperature of the lipids) to produce large unilamellar vesicles (LUV). The final total concentration of lipids was 250 μM in 200 μl. Compounds (or 0.5% DMSO) and 1 mM $CaCl_2$ or 1 mM EDTA (negative control) were added to the vesicles, and the reaction was started by addition of 500 ng his-tagged $cPLA_{2\alpha}$ (in 10 μl buffer). After 1 h at 37 °C, 1.6 ml methanol was added together with 1 nmol γ-linolenic acid, 40 μl 1N HCl and

1.6 ml D-PBS pH 7.4. The fatty acids were extracted with solid phase C_{18} columns (100 mg, UCT, Bristol, PA, USA) which were conditioned with 1 ml methanol and 1 ml D-PBS pH 3. After a washing step with 1 ml H_2O and 1 ml 70% methanol, the fatty acids were eluted with 1 ml methanol. Following derivatization with p-anisidinium chloride (derivatisation mixture: 0.75 mM *p*-anisidinium chloride, 9.375 mM 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide hydrochloride and 0.11% pyridine), the resulting derivative was extracted by solid phase extraction after addition of 900 μl H_2O. C_{18} columns were conditioned with 1 ml methanol and 1 ml H_2O and the samples washed with 1 ml acid methanol 50%(200 μl 1 N HCl/100 ml) and 1 ml methanol 50% and eluted with 300 μl methanol. After addition of 60 μl H_2O, 50 μl of the samples was analyzed by RP-HPLC at 249 nm as described (Hoffmann et al., 2010). In brief, derivatized AA was eluted on a C_{18} column (Nova-Pak cartridge, 5 × 100 mm, 4 μm particle size, Waters, Eschborn, Germany) with gradient elution starting from methanol/TFA (85/0.007) to methanol/TFA (100/0.007). The amount of the released AA was quantified after peak area integration at the basis of γ-linolenic acid as internal standard.

3.2.14 Induction of mPGES-1 expression in A549 cells and preparation of microsomes

Preparations of A549 cells and determination of mPGES-1 activity was performed as described (Koeberle et al., 2008b). In brief, cells were seeded with a cell density of 1×10^5/ml in DMEM/High glucose (4.5 g/l)/stable L-glutamine, penicillin (100 U/ml)/streptomycin (100 μg/ml) and 10% FCS. After 20 h, the medium was exchanged by medium containing 2% FCS and stimulated with 2 ng/ml IL-1β in DMEM for 72 h at 37 °C and 6% CO_2. Cells were harvested by trypsination, centrifuged (600 × g/10 min/20 °C) and washed with D-PBS pH 7.4. The cell pellet was frozen in liquid nitrogen (1 min). Then, the frozen pellet was resuspended in homogenization buffer (0.1 M potassium phosphate buffer, pH 7.4, 1 mM PMSF, 60 μg/ml STI, 1 μg/ml leupeptin, 2.5 mM GSH, and 250 mM sucrose) and sonicated (3 × 20 s). The homogenate was subjected to differential centrifugation (10,000 × g/10 min/4 °C). The resulting supernatant was centrifuged at 174,000 × g for 1 h at 4 °C. The pellet (microsomal fraction) was resuspended in 1 ml homogenization buffer and the total protein concentration was determined with a modified Bradford assay (Roti®Nanoquant, Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Aliquots were frozen at −80 °C until use.

3.2.15 Determination of PGE_2 formation from microsomal preparations of A549 cells

Microsomal membranes were diluted in homogenization buffer depending on the amount of protein (about 300 μg protein/ml). Test compounds or vehicle (DMSO) were added to microsomal preparations of A549. After 15 min at 4 °C, the reaction (100 μl total volume) was initiated by addition of PGH_2 at the indicated concentrations. After 1 min at 4 °C, the reaction was terminated using stop solution (100 μl; 40 mM FeCl2, 80 mM citric acid and 10 μM 11β-PGE_2 as internal standard). PGE_2 was separated by solid-phase extraction with C_{18} columns (100 mg, UCT, Bristol, PA, USA) which were conditioned with acetonitrile and water. After washing with 2 × 400 μl H_2O, PGE_2 was eluted with 200 μl acetonitrile and 400 μl H_2O was

added to the eluate. PGE_2 formation was quantified by RP-HPLC on a C_{18} column (Nova-Pak cartridge, 5 × 100 mm, 4 μm particle size, Waters, Eschborn, Germany) with acetonitrile/H_2O/TFA (30/70/0.007) at 195 nm.

3.2.16 Determination of PGE_2 and 6-keto $PGF_{1\alpha}$ in LPS stimulated monocytes

Monocytes (10^6/ml in RPMI 1640 containing penicillin (100 U/ml)/streptomycin (100 μg/ml), 2 mM L-glutamine and 2% human serum (1 ml per sample in a 12-well plate). After 1.5 h, 37 °C, 6% CO_2 cells were stimulated with 1 μ/ml LPS for 20 h, 37 °C, 6% CO_2. After 3 washing steps with PBS, medium was added for 30 min and renewed again. Inhibitors or DMSO (0.1% as vehicle) were added for 15 min, 37 °C, 6% and cells stimulated for 30 min with 1 μM AA. The supernatant was collected and centrifuged to remove cells and frozen at −20 °C until analysis. For PGE_2 analysis samples were diluted 1:2 with assay buffer and the EIA was performed according to manufacturer's instructions (Biotrend Chemikalien GmbH (Köln, Germany). For 6-keto $PGF_{1\alpha}$ analysis samples were diluted 1:5 with assay buffer and the EIA was performed according the manufacturer's instructions (Sapphire Bioscience (Waterloo, Australia)).

3.2.17 Determination of PGE_2 in LPS stimulated human blood

Heparinized human blood was preincubated with inhibitors or DMSO (0.1% as vehicle) (15 min, 37 °C) and the blood stimulated for 24 h with 10 μg/ml LPS at 37 °C. The reaction was stopped on ice and the blood centrifuged (600 × g/ 10 min/ 4 °C). The plasma was collected and frozen at −20 °C. PGE_2 concentration was quantified in 1:100 diluted samples with EIA according to manufacturer's instructions (Biotrend Chemikalien GmbH (Köln, Germany)).

3.2.18 Determination of 6-keto $PGF_{1\alpha}$ formation in interleukin-1β stimulated A549 cells

For the induction of COX-2 expression, A549 cells were seeded with a cell density of 1 × 10^5/ml in DMEM/High glucose (4.5 g/l)/stable L-glutamine, penicillin (100 U/ml)/streptomycin (100 μg/ml) and 10% FCS. After 20 h the medium was exchanged by DMEM/ High glucose (4.5 g/l)/ stable L-glutamine, penicillin (100 U/ml)/streptomycin (100 μg/ml) and 2% FCS and stimulated with 2 ng/ml IL-1β in DMEM for 72 h at 37 °C and 6% CO_2. After trypsination, cells were washed twice with D-PBS pH 7.4 and resuspended in PGC buffer (2 × 10^6/ml). A549 were incubated with compounds or vehicle (0.1% DMSO) for 15 min at 37 °C. Formation of 6-keto $PGF_{1\alpha}$ was induced by addition of 3 μM AA for 15 min at 37 °C. The incubation was stopped on ice for 5 min. The supernatants were collected after centrifugation (800 × g/5 min/4 °C) and stored at −80 °C. 6-Keto $PGF_{1\alpha}$ was quantified with EIA according to the manufacturer's (Sapphire Bioscience (Waterloo, Australia)) instructions.

3.2.19 Determination of [^{3}H]-AA-release in PMNL and monocytes

For the determination of AA release in PMNL and monocytes, cells were incubated with tritium labeled AA. PMNL (5 $\times$ 10^6/ml) and monocytes (2 $\times$ 10^6/ml) were incubated with 0.5 μCi [^{3}H]-AA for 2 h at 37 °C in RPMI 1640 without additives. Cells were washed twice with PG buffer containing 2 mg/ml BSA, faf. Cells were resuspended in PGC, PGC containing BSA, faf or plasma as indicated. For inhibitor studies, cells were preincubated with compounds or vehicle (0.1% DMSO) for 15 min at 37 °C. Cells were stimulated with A23187 in the indicated concentrations. After 5 min the reaction was stopped on ice for 10 min. 300 μl of the supernatant was collected after centrifugation at 500 $\times$ g/15 min/20 °C and added to 2 ml LSC fluid (Rotiszint Eco Plus®, Carl Roth GmbH & Co. KG, Karlsruhe, Germany). The released [^{3}H]-AA was measured on a scintillation counter (PerkinElmer LifeSciences, Waltham, Massachusetts, USA).

3.2.20 Generation of whole cell lysate

Granulocytes (10^7/100 μl ice-cold PG buffer) were mixed 2:1 with ice-cold 4 $\times$ sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer (50 μl, 40 mM Tris-HCl, pH 8, 4 mM EDTA, 10% (m/v) SDS, 10% (v/v) β-mercaptoethanol), heated for 6 min at 95 °C, sonicated (3 $\times$ 10 s) at 4 °C and analyzed for 5-LO protein by SDS-PAGE and immunoblotting.

3.2.21 Determination of subcellular localization and redistribution of 5-lipoxygenase

For the analysis of the subcellular localization of the 5-LO enzyme, 3 $\times$ 10^7 PMNL/ml PGC buffer were preincubated with compounds or vehicle (0.1% DMSO) for 15 min at 37 °C. After stimulation with 2.5 μM A23187 for 5 min at 37 °C, cells were chilled on ice and centrifuged at 200 $\times$ g/5 min/4 °C. For the subcellular fractionation by mild detergent lysis PMNL were resuspended in 0.1% Nonidet P-40 lysis buffer (Werz et al., 2001b) (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 0.1% NP40, 1 mM PMSF, 60 μg/ml STI, and 10 μg/ml leupeptin). After vortexing for 3 $\times$ 5 s and incubation on ice for 10 min, samples were subsequently centrifuged (1,000 $\times$ g/10 min/4 °C) (Pouliot et al., 1996). The supernatant (non-nuclear fraction) was collected and the pellet (nuclear fraction) was washed with relaxation buffer (50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF, 60 μg/ml STI, and 10 μg/ml leupeptin). The nuclear fraction was resuspended in 200 μl relaxation buffer and sonified 3 $\times$ 10 s to disrupt the nuclei. Disruption of cellular membranes and integrity of nuclei was analyzed under the light microsope mixing the samples with trypanblue. Equal amounts (100 μl) of nuclear and non-nuclear fraction were mixed with 2 $\times$ SDS loading buffer (100 μl, 20 mM Tris-HCl, pH 8, 2 mM EDTA, 5% (m/v) SDS, 10% (v/v) β-mercaptoethanol) and samples were boiled for 6 min at 95 °C. Samples were analyzed for 5-LO protein by SDS-PAGE and western blot. Lamin B was also detected to verify the correct separation into subcellular fractions (not shown).

3.2.22 Determination of ERK phosphorylation

Human PMNL (10^7/100 μl PGC buffer) were preincubated with compounds or vehicle (1% DMSO) for 15 min at 37 °C followed by stimulation with 1 μM fMLP for 1.5 min at 37 °C. The reaction was stopped by addition of 100 μl of 2 × SDS loading buffer. Samples were vortexed, boiled for 6 min at 95 °C and sonicated (3 × 10 s) at 4 °C. Phosphorylated ERK1/2 was analyzed in total cell lysates by SDS-PAGE and Western Blot.

3.2.23 SDS-PAGE and Western Blot

For the analysis of proteins by SDS-PAGE and western blot, samples from total cell lysates were mixed with 10 μl 0.1% bromophenol blue (BPB) and glycerol (1:1, v/v). Aliquots from nuclear and non-nuclear fraction (200 μl) were mixed with 80 μl 0.1% BPB and glycerol (1:1, v/v). Samples from ERK phosphorylation assays (200 μl) were mixed with 40 μl 0.1% bromophenol blue (BPB) and glycerol (1:1, v/v). 10 μl of each sample was loaded on a gel (10% or 16% polyacrylamide) and the proteins were separated with a Mini Protean system (Bio-Rad Laboratories Inc., Hercules, CA, USA)(Shapiro et al., 1967). The pre-stained marker peqGOLD IV (10-170 kDa) (peqLab Biotchenology, Erlangen Germany) was used for evaluation of the molecular weight of the proteins. Separated proteins were electroblotted on nitrocellulose membranes (Amersham Hybond™-C Extra, GE Healthcare, Munich, Germany) with tank blotting method (Bio-Rad Mini Trans-Blot® cell, Bio-Rad Powerpac™Basic, Bio-Rad Laboratories Inc., Hercules, CA, USA) at 90 V for 90 min in transfer buffer (48 mM Tris, 40 mM glycine, 0.1 mM SDS, 20% methanol (v/v)). Correct loading and transfer of the proteins was confirmed by Ponceau Staining (5% Ponceau S in 5% acetic acid). Membranes were blocked with 5% BSA (w/v) in TBS-Tween (tris buffered saline (TBS): 50 mM Tris/HCl, pH 7.4, 100 mM NaCl; and 0.1% Tween-20®) for 1 h at room temperature. Membranes were washed with TBS-Tween and incubated over night at 4 °C with primary antibodies as indicated (Table 3.3). After extensively washing with TBS-Tween, membranes were incubated with secondary antibodies. Detection of proteins was performed by the use of alkaline phosphatase-conjugated IgGs (1:1,000 dilution) (Sigma-Aldrich, Taufkirchen, Germany) followed by visualization with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) in detection buffer (100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) and acquisition and analysis with the ImageQuant software (GE healthcare) or with infrared-labeled secondary antibodies IRDye 800CW (1:10,000 dilution) or 680LT (1:40,000 dilution) (LI-COR Biosciences, Lincoln, NE, USA), detection with an Odyssey Infrared Imaging System (LI-COR Bioscience, Lincoln, NE, USA) and analysis by the Odyssey application software (version 3.0.25).

Table 3.3: Primary antibodies. Antibodies were diluted in 5% BSA (w/v) in TBS-Tween.

primary	source	dilution	company
5-LO	mouse	1:8	Prof. Steinhilber (Goethe University, Frankfurt am Main, Germany)
Lamin B	mouse	1:200	Abcam (Cambridge, UK)
pERK	mouse	1:1000	Cell Signaling Technology, Inc. (Danvers, MA, USA)
ERK	rabbit	1:1000	Cell Signaling Technology, Inc. (Danvers, MA, USA)
β-Actin	rabbit	1:1000	Cell Signaling Technology, Inc. (Danvers, MA, USA)

3.2.24 Viability assays

To investigate effects of the compounds on viability of the cells, trypan blue exclusion was used to study short term effects (30 min) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for long term incubations (20 to 48 h) (Mosmann, 1983). Trypan blue exclusion was either determined in monocytes or PMNL (5×10^6/ml PG buffer) after incubation with compounds or vehicle (0.1% DMSO) for 30 min at 37 °C. Cells were mixed with a trypan blue solution (1:1, 0.2%) and counted by a Vi-cell counter (Beckmann Coulter GmbH, Krefeld). For the MTT assay, cells were seeded in 96-well plates (100 μl) in the indicated concentrations and incubated with compounds or vehicle (0.3% DMSO) for 20 to 48 h at 37 °C, 6% CO_2. MTT (20 μl of 5 mg/ml in D-PBS pH 7.4) was added for 2 to 4 h at 37 °C, 6% CO_2 and the formed formazan was solubilized by addition of 100 μl SDS-lysis buffer (20 mM HCl (pH 4.5), 10% SDS (w/v)). After incubation with extensive shaking in the dark for 16 h at room temperature, the absorbance at 595 nm was measured using a Victor^3 plate reader (Perkin Elmer, Rodgau-Jügesheim, Germany) or a Multiskan Spectrum Reader (Thermo Fisher Scientific Oy, Vantaa, Finland).

3.2.25 Determination of the formation of reactive oxygen species in neutrophils

Formation of reactive oxygen species (ROS) in PMNL was measured by two different methods. In Tuebingen, a spectrofluorometer equipped with a cuvette was used for the samples of the pregnancy project. In Jena, a 96-well approach was established applying a microplate reader to study compounds and the effect of plasma on PMNL. For the cuvette method, PMNL (5×10^6/ml ice-cold PG buffer) were prewarmed for 3 min at 37 °C. Then, the peroxide-sensitive dye 2',7'-dichlorofluorescein-diacetate (DCF-DA) (1 μg/ml) was added to the cells for 2 min at 37 °C and 1 mM $CaCl_2$ was added for 1 min at 37 °C under stirring. The reaction was started by addition of 1 μM ionomycin or 0.1 μM PMA for 5 min at 37 °C. Ionomycin was used as Ca^{2+} ionophore instead of A23817, because A23187 interfered with the detection of ROS. The fluorescence emission (530 nm) was measured after excitation at 485 nm in a spectrofluorometer (Amicon-Bowman series 2, Thermo Spectronic, Rochester, NY, USA).
For the 96-well method, PMNL (10^7/ml PG buffer) were preincubated with compounds (or 0.1% DMSO as vehicle) or plasma for 15 min at 37 °C. Then, DCF-DA (1 μg/ml) and $CaCl_2$ (1 mM) were added 2 min prior addition of 0.1 μM PMA or

0.1 μM fMLP. The fluorescence emission at 530 nm was measured after excitation at 485 nm in a thermally controlled (37 °C) NOVOstar microplate reader (BMG Labtechnologies GmbH, Offenburg, Germany).

3.2.26 Activity assay of isolated COX-1 and COX-2

Inhibition of the activities of isolated ovine COX-1 and human COX-2 was performed as described (Koeberle et al., 2008a). Briefly, purified COX-1 (ovine, 50 units) or COX-2 (human recombinant, 20 units) were diluted in 1 ml reaction mixture containing 100 mM Tris buffer pH 8, 5 mM GSH, 5 μM hemoglobin, and 100 μM EDTA at 4 °C and pre-incubated with the test compound for 5 min. Samples were pre-warmed for 60 s at 37 °C, and AA (5 μM for COX-1, 2 μM for COX-2) was added to start the reaction. After 5 min at 37 °C, the reaction was stopped, PGB_1 added as standard and 12-HHT was extracted and then analyzed by HPLC (Albert et al., 2002).

3.2.27 Intracellular calcium measurements

For the measurement of intracellular calcium, PMNL (10^7/ml in PG buffer) were prestained with Ca^{2+} sensitive dye Fura-2/AM (2 μM) for 45 min at 37 °C in the dark. After two washing steps, cells were resuspended in PG buffer containing 0.01% BSA at a density of 5×10^6/ml. 100 μl of cell suspension was pipetted into a 96 well plate and the cells were preincubated for 10 min at 37 °C with the respective inhibitors or 1% DMSO as control. 2 min prior to stimulation 1 mM $CaCl_2$ was added. Cells were stimulated with 0.1 μM fMLP and the signal monitored after emission at 510 nm after excitation at 340 nm (Ca^{2+} bound Fura-2) and 380 nm (free Fura-2) in a thermally controlled (37 °C) NOVOstar microplate reader (BMG Labtechnologies GmbH, Offenburg, Germany). After cell lysis with Triton X-100 the maximal fluorescence signals could be monitored and after chelating Ca^{2+} with 10 mM EDTA the minimal fluorescence signals. The amount of Ca^{2+} was calculated from the ratio of the signals at 340 and 380 nm according to (Grynkiewicz et al., 1985).

3.2.28 LC-MS/MS analysis

Liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses were carried out on an Acquity Ultraperformance LC (UPLC) BEH C_{18} column (1.7 μm, 2.1 $\times$ 50 mm, Waters, Milford, MA) using an Acquity^{TM}UPLC system (Waters, Milford, MA, USA) and a QTRAP 5500 Mass Spectrometer (AB Sciex, Darmstadt, Germany) equipped with a Turbo V^{TM}Source and electrospray ionization (ESI) probe. LO products (4 μl injection) were separated at a flow rate of 0.8 ml/min and a column temperature of 45 °C. The solvents for the mobile phase were acetonitrile (A) and water/acetonitrile (90/10; B) both acidified with 0.07% (v/v) formic acid. Isocratic elution at A/B = 30% was performed for 2 min, and followed by a linear gradient to 70% B within 5 min. LO products were detected by multiple reaction monitoring in the negative ion mode using a dwell time of 10 ms. The ion spray voltage was set to 4500 V, the heater temperature to 500 °C, the declustering potential to 50-120 eV, the entrance potential to 10 eV and the collision cell exit potential to 11-22 eV, the spray gas pressure to 50 psi, the Turbo V gas pressure to 80 psi and the curtain gas pressure to 20 psi. Monitored transitions of LO products and their collision

energies are given in Table 3.4. The transition first mentioned ('transition 1') was used for quantification. Automatic peak integration was performed with Analyst 1.6 software (AB Sciex, Darmstadt, Germany) using IntelliQuan default settings. Data were normalized on the internal standard PGB_1 and are given as relative intensities. The reported method was optimized for analysis of the comparison of LO products between pregnant and non-pregnant samples and not for absolute quantification.

Table 3.4: MS conditions for multiple reaction monitoring. [1]transition used for quantification, [2]first quadrupol, [3] third quadrupol.

compound	transition 1[1]			transition 2		
	Q1 (m/z)[2]	Q3 (m/z)[3]	collision energy (eV)	Q1 (m/z)[2]	Q3 (m/z)[3]	collision energy (eV)
PGB_1	335	113	31	335	221	28
LTB_4	335	129	26	335	195	22
5-HETE	319	115	20	319	203	20
8-HETE	319	155	18	-	-	-
11-HETE	319	167	21	-	-	-
12-HETE	319	179	18	-	-	-
15-HETE	319	219	18	-	-	-

3.2.29 Determination DPPH scavenging activity

The radical scavenger capability was assessed by measuring the reduction of the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) as described (Blois, 1958). Briefly, 100 μl of 5, 25, 50 μM solutions of compounds (in DMSO, corresponding to 0.5, 2.5, and 5 nmol) were added to 100 μl of a solution of the stable free radical DPPH in ethanol (50 μM, corresponding to 5 nmol), buffered with acetate to pH 5.5, in a 96-well plate. The absorbance was recorded at 520 nm (Multiskan Spectrum Reader, Thermo Fisher Scientific Oy, Vantaa, Finland) after 30 min incubation under gentle shaking in the dark. Ascorbic acid and L-cysteine were used as reference compounds (H_2O as vehicle).

3.2.30 Animal models of inflammation

The animal experiments were conducted at the group of Prof. Bruno D'Agostino at the Department of Experimental Medicine, University of Naples, Italy. For this male adult CD1 mice (25-35 g, Harlan, Milan, Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care complied with Italian regulations on protection of animals used for experimental and other scientific purpose (Ministerial Decree 116192) as well as with the European Economic Community regulations (Official Journal of E.C. L 358/1 12/18/1986). Mice were lightly anesthetized with enflurane and received subplantar injection of 50 μl of carrageenan 1% (w/v). Paw volume was measured using a hydroplethismometer specially modified for small volumes (Ugo Basile, Milan, Italy) immediately before the subplantar injection (basal value) and 2, 4, 6, 24, 48, and 72 h thereafter. Mice

were divided in 7 groups (n=6) and received *i.p.* administration of compound RF-Id (0.1, 1, and 10 mg/kg) or vehicle (DMSO), 30 min before carrageenan application. Mice (28-30 g, two groups, n=6, each) received *i.p.* administration of compound RF-Id (0.1 mg/kg) or vehicle (DMSO), 30 min before induction of inflammation. Mice were then lightly anesthetized with enflurane. Air pouches were developed by subcutaneous injection of 2.5 ml sterile air into the back of mice. Three days later, 2.5 ml of sterile air was re-injected in the same cavity. After another three days, 1 ml of zymosan 1% (w/v) or vehicle (saline) was injected into the air pouch, and after another 4 h, mice were sacrificed by CO_2 exposure and exudate in the pouch was collected with 1 ml of saline and placed in graduated tubes and centrifuged at 125 $\times$ g for 10 min. The pellet was suspended in 500 μl of saline and leukocytes were evaluated by optical microscopy in the cell suspension diluted with Turk's solution.

3.2.31 Statistics

Results are expressed as mean $\pm$ standard error (SEM) of the mean of n observations, where n represents the number of paired experiments. Statistical evaluation of the data was conducted using GraphPad Prism software (San Diego, CA), and was performed by Student's t test for paired observations and one-way ANOVA followed by a Bonferroni ($<$ 5 groups) or Tukey-Kramer ($<$ 5 groups) post-hoc test for multiple comparisons, respectively.. The tests were conducted using a two-sided alpha level of 0.05 (*p $<$ 0.05). For evaluation of normal distribution of data, the Kolmogorov-Smirnov test was used. IC_{50} values were calculated from averaged measurements at 3-5 different concentrations of the compounds by nonlinear regression using GraphPad Prism software (San Diego, CA) one site binding competition.

4 Results

4.1 Influence of pregnancy on LO derived product formation

In order to study the influence of pregnancy on LT formation data was randomly collected from blood of pregnant donors and corresponding non-pregnant females. Parts of this study were published in (Schaible et al., 2013a). As shown in Table 4.1, the gestational age of the analyzed healthy pregnant women was between weeks 15 to 36. The pregnant donors were distributed almost equally in the 2nd (13th to 24th week, 14 donors) and the 3rd (25th to 36th week, 13 donors) trimester.

Table 4.1: Characteristics of the donors. Data are presented as median and range.

	pregnant	non-pregnant
subjects (n)	27	26
age (yrs)	29 (24-36)	25 (20-30)
gestational week	24 (15-36)	-
cycle day	-	13 (3-32)

4.1.1 Higher leukotriene formation during pregnancy

5-LO product formation in blood from pregnant and non-pregnant females was compared pair-wise. Following stimulation with Ca^{2+}-mobilizing agent A23187 the formation of 5-LO products (LTB_4, its trans-isomers and 5-H(p)ETE) was higher in blood derived from pregnant versus non-pregnant females (about 2-fold , 95% CI: 1.4-2.7 (A23187)) (Fig. 4.1A). Applying bacterial products LPS and fMLP a similar effect was observed (about 3-fold, 95% CI: 1.5-4.4 (fMLP)) (Fig. 4.1B, left panel). This was evident for both LTB_4 formation as well as 5-H(p)ETE formation (Fig. 4.1A and B). Subdividing the data between second and third trimester did not show any significant differences between the trimesters though the effect is higher during the second trimester (Fig. 4.1C). Substitution of substrate AA to the blood strongly induced the amount of product formed by both pregnant as well as non-pregnant derived blood. Interestingly however, the difference between blood from pregnant and non-pregnant donors was reduced (about 1.4 fold; 95% CI: 1.1-1.7) (Fig. 4.1B, right panel).

Then other LO products such as 12-H(p)ETE and 15-H(p)ETE were analyzed after stimulation with A23187 (plus substrate AA) in the blood from pregnant and non-pregnant donors (Fig. 4.2). 12-LO derived 12-H(p)ETE was significantly higher in blood from pregnant compared to non-pregnant donors after stimulation with A23187 only (Fig. 4.2A, left panel) and no difference was observed analyzing the trimesters separately (Fig. 4.2A, right panel). After application of substrate this

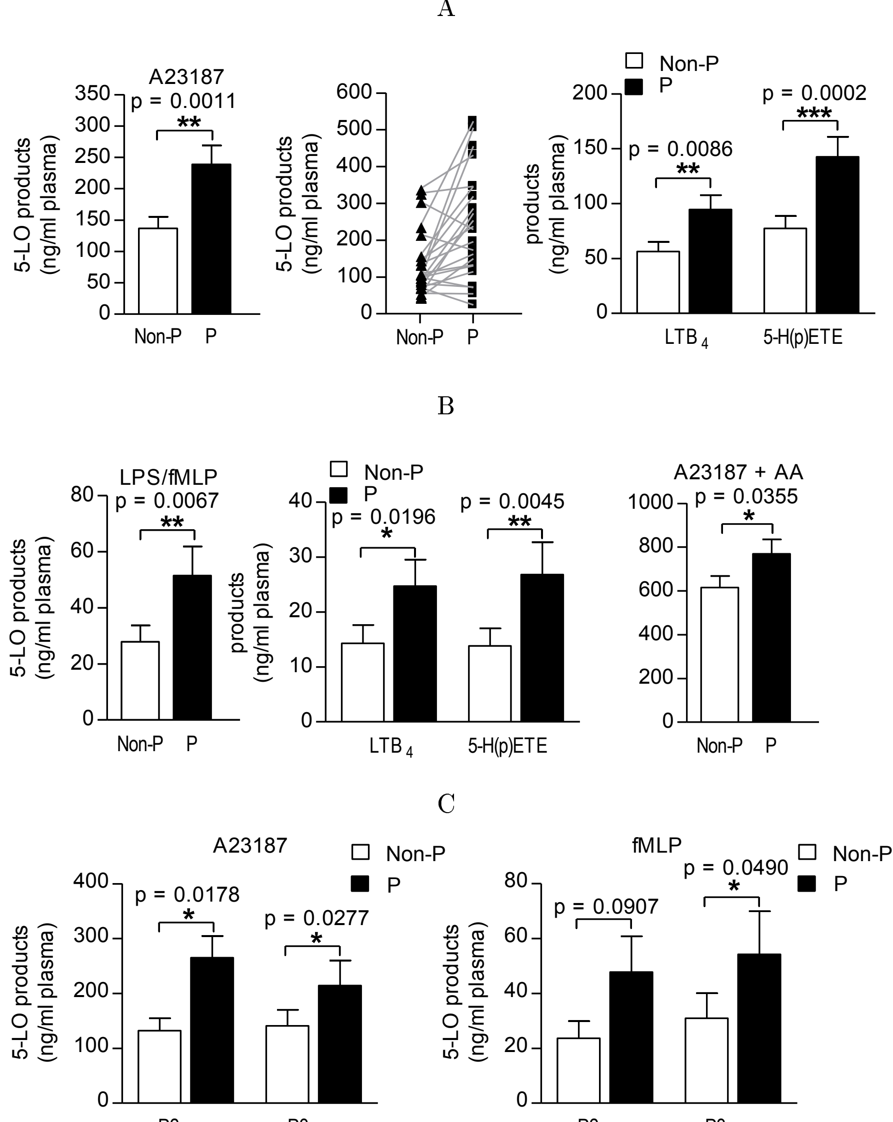

Figure 4.1: Higher LT formation in blood from pregnant donors. (A) 5-LO product formation in blood from pregnant (P) and non-pregnant (Non-P) donors induced by A23187 (30 μM, 10 min, 37 °C). In (A), middle panel, lines connect dots corresponding to the pair-wise Non-P/P analyses at different experimental days. In the right panel, LTB_4 and 5-H(p)ETE were analyzed separately. (B) 5-LO product formation in blood stimulated with fMLP (1 μM, 15 min, 37 °C, left and middle panel) after priming with LPS (1 μg/ml, 30 min, 37 °C) and with A23187 plus AA (30 μM and 100 μM, respectively, 10 min, 37 °C, right panel). In the middle panel, LTB_4 and 5-H(p)ETE were analyzed separately. Data are means + SEM; n = 23 (A23187), n = 19 (fMLP/LPS), n = 19 (A23187 plus AA). (C) 5-LO product formation in blood from pregnant and non-pregnant donors induced by A23187 (left panel) and fMLP (right panel) separated into trimesters. Data are means + SEM; n = 11 (A23187, 2nd trimester (P2)), n = 12 (A23187, 3rd trimester (P3)), n = 8 (fMLP, P2), n = 11 (fMLP, P3). Data passed normality Kolmogorov-Smirnov test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; pregnant vs. non-pregnant, paired t-test.

difference was no longer evident (Fig.4.2B, left panel). 15-H(p)ETE formation was not different between pregnant and non-pregnant donors (Fig. 4.2, right panel). 12-HHT as COX-1 product was also analyzed in the blood. Here, no difference between pregnant and non-pregnant blood was observed regardless the stimulus used (Fig. 4.2C).

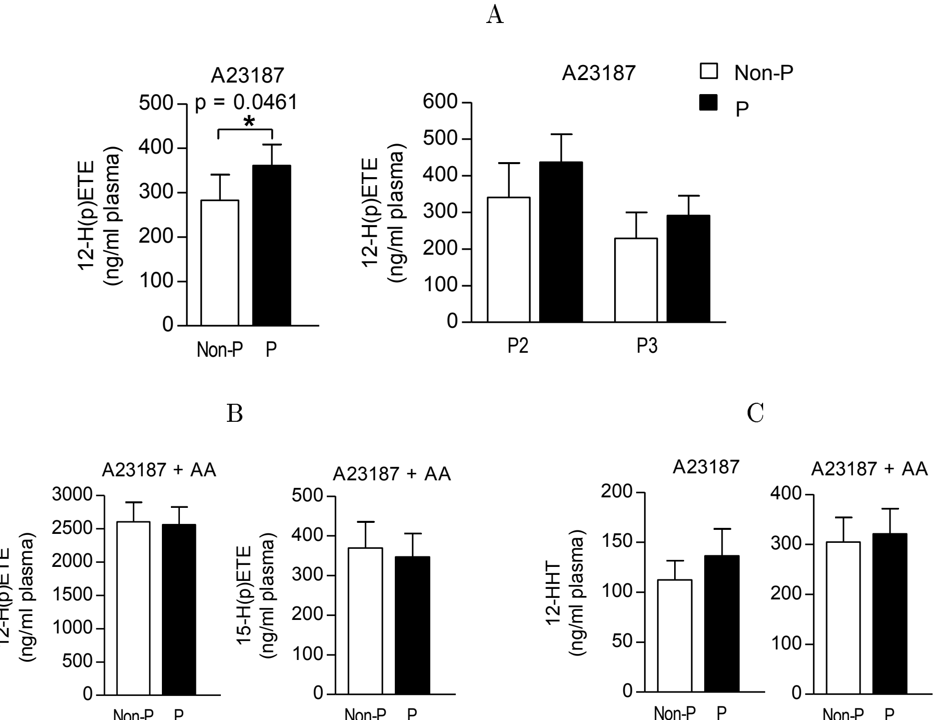

Figure 4.2: Analyses of diverse LO products 12-H(p)ETE, 15-H(p)ETE and COX-1 product 12-HHT in blood from pregnant donors. (A) 12-H(p)ETE formation in blood from pregnant (P) and non-pregnant (Non-P) donors after stimulation with A23187 (30 μM, 10 min, 37 °C, left panel). Data are means + SEM; n = 23. 12-H(p)ETE formation in blood from pregnant and non-pregnant donors induced by A23187 separated into trimesters (right panel). Data are means + SEM; n = 11 (2nd trimester (P2)), n = 12 (3rd trimester (P3)). (B) 12-H(p)ETE and 15-H(p)ETE formation in blood from pregnant and non-pregnant donors stimulated with A23187 plus AA (30 μM and 100 μM, respectively, 10 min, 37 °C). Data are means + SEM, n = 19 (12-H(p)ETE); n = 19 (15-H(p)ETE). (C) COX-1 product 12-HHT in blood from pregnant and non-pregnant donors stimulated with A23187 (30 μM, 10 min, 37 °C, left panel) and A23187 plus AA (30 μM and 100 μM, respectively, 10 min, 37 °C, right panel). Data are means + SEM; n = 14 (A23187), n = 14 (A23187 plus AA). Data passed normality Kolmogorov-Smirnov test; *, $p < 0.05$; pregnant vs. non-pregnant, paired t-test.

To strengthen the data collected after analysis with HPLC-UV, a LC-MS/MS method was applied which confirmed the results observed. As shown in Fig. 4.3, 5-LO derived products were higher in samples from pregnant donors compared to non-pregnant donors after stimulation, however not significantly. By this, also AA derived products like 8-HETE and 11-HETE could be analyzed. For all LO derived products other than from 5-LO, no difference was observed between blood from pregnant and non-pregnant donors. In unstimulated controls, however, there was a tendency for higher amounts in the non-pregnant derived samples (8-, 11- and 15-HETE).

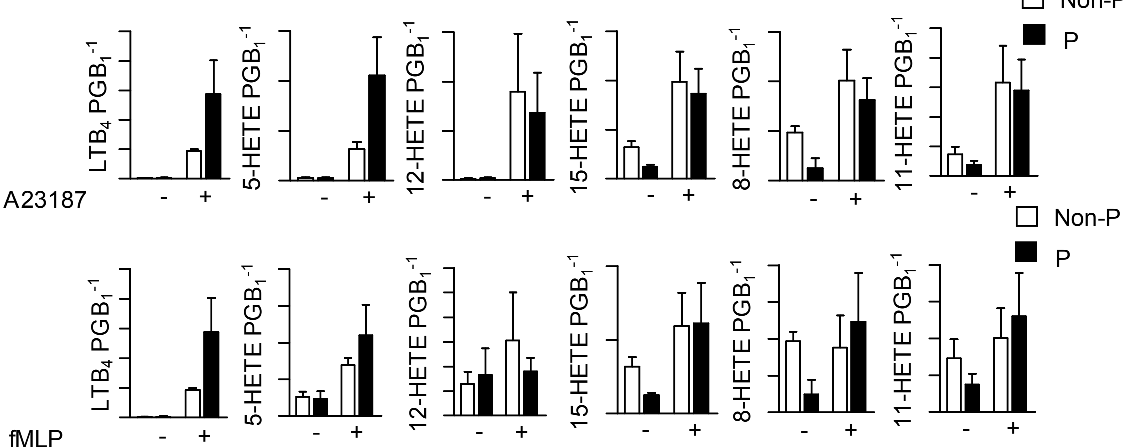

Figure 4.3: LC-MS analysis of LTs in blood from pregnant and non-pregnant females. Product formation in blood from pregnant (P) and non-pregnant (Non-P) donors induced by A23187 (30 μM, 10 min, 37 °C, upper panel) and fMLP (1 μM, 15 min, 37 °C) after priming with LPS (1 μg/ml, 30 min, 37 °C, lower panel). Data are expressed as + SEM. n = 3.

4.1.2 Blood cell counts and blood parameters

Higher amounts of LTs present in stimulated blood from pregnant donors might originate from higher cell numbers of LT forming cells (neutrophils and monocytes, (Werz, 2002). In accordance with other studies (Minagawa et al., 1999; Luppi et al., 2002b) leukocyte numbers were increased in samples from pregnant donors whereas erythrocyte numbers were lower and platelet numbers were not influenced by pregnancy (Fig. 4.4). The numbers of LT synthesizing neutrophils and also monocytes were significantly elevated comparing samples from pregnant and non-pregnant females (1.9 fold, 95% CI: 1.6-2.2 (neutrophils), 1.4 fold, 95% CI: 1.2-1.6 (monocytes)) (Fig. 4.4), both considering absolute as well as relative cell numbers (not shown). No significant differences were found for basophils and lymphocytes. Eosinophil numbers were slightly lower in samples from pregnant donors (0.8 fold, 95% CI: 0.48-1.1) in both, 2nd and 3rd trimester. Other blood parameters such as hematocrit, hemoglobin concentration, mean cellular hemoglobin (MCH), mean cellular hemoglobin concentration (MCHC) and mean cell volume (MCV) were as expected. Hematocrit as well as hemoglobin concentration were significantly lower in samples from pregnant compared to non-pregnant donors. MCHC was higher in samples from pregnant donors. MCH and MCV were not different (Fig. 4.4) (Lurie and Mamet, 2000). Assuming that the capacity of isolated granulocytes and monocytes to form LTs is the same between cells in the blood from pregnant and non-pregnant donors, the higher number of granulocytes and monocytes might account for the higher LT formation from maternal blood. Therefore, the capacity to synthesize LTs was analyzed in isolated granulocytes and of PBMC, next.

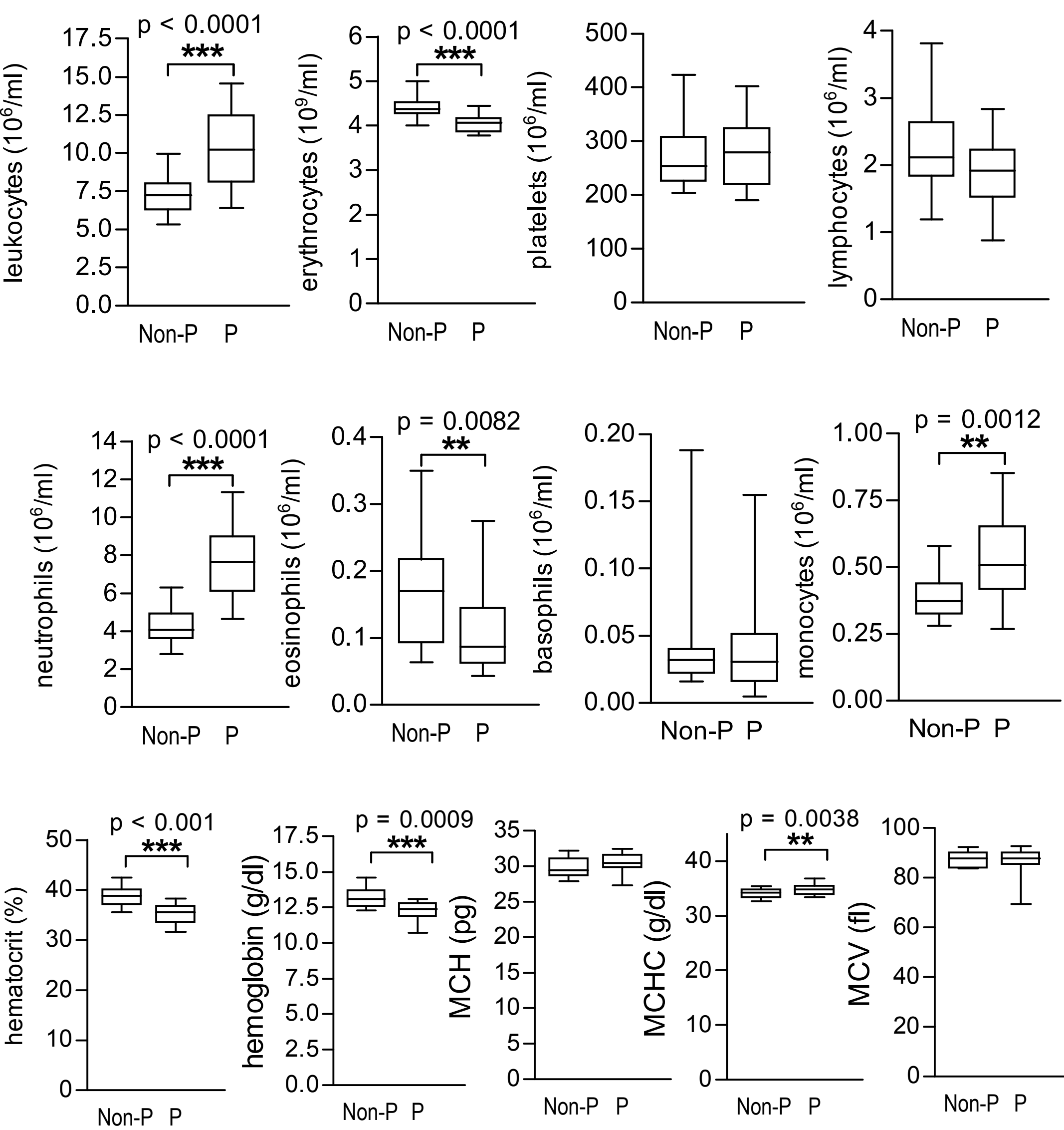

Figure 4.4: Blood cell counts. Data are expressed as 25th, 50th and 75th percentiles and the range; n = 19; Data passed normality Kolmogorov-Smirnov test;**, $p < 0.01$; ***, $p < 0.001$; pregnant (P) vs. non-pregnant (Non-P), paired t-test. Mean cellular hemoglobin (MCH), mean cellular hemoglobin concentration (MCHC) and mean cell volume (MCV).

4.1.3 Leukotriene formation in isolated granulocytes

Interestingly, stimulation of isolated granulocytes with A23187 led to lower LT formation in cells derived from blood of pregnant compared to non-pregnant females (about 75%, 95% CI: 64-87%; Fig. 4.5A, left panel). This difference was observed in samples from the 2nd as well as the 3rd trimester (Fig. 4.5A, right panel). After substitution of substrate AA, this difference was reduced but still evident (Fig. 4.5B, left panel, about 95%; 95% CI: 79-112%), with a stronger reduction in samples from the 3rd trimester (Fig. 4.5B, right panel). Both LT synthesis capacity as well as protein amount, were slightly lower in neutrophil homogenate samples from pregnant compared to non-pregnant donors (Fig. 4.5D and E). Preliminary experiments regarding the release of AA induced by A23187 revealed no difference between cells from pregnant and non-regnant donors (Fig. 4.5C). In summary, the results depict

that the lower capacity of granulocytes to form LTs is not only depending on a lower amount of active enzyme in granulocytes derived from pregnant donors but also on others factors.

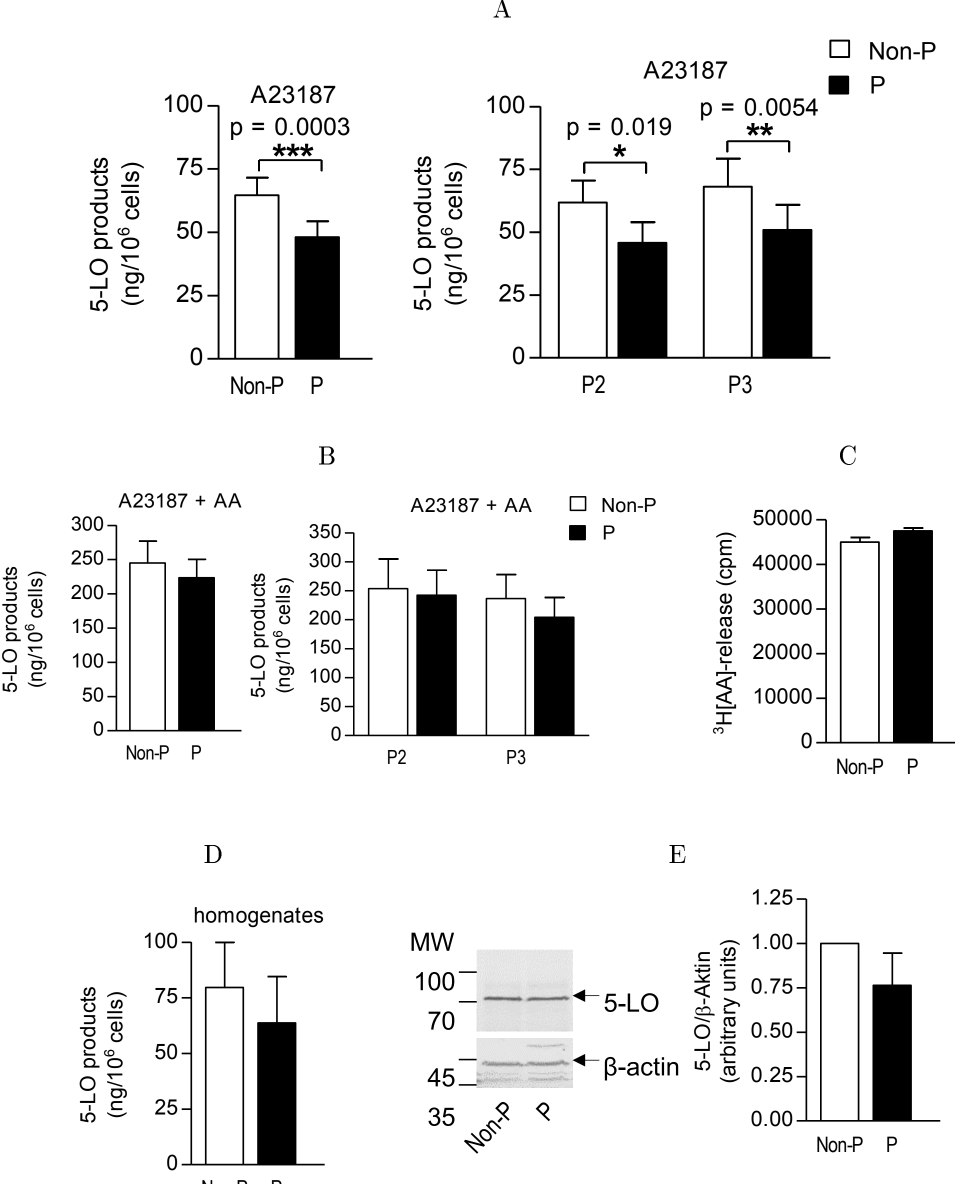

Figure 4.5: 5-LO activity is lower in isolated granulocytes from pregnant vs. non-pregnant donors. (A) Granulocytes from pregnant (P) and non-pregnant (Non-P) donors stimulated with A23187 (2.5 μM, 10 min, 37 °C, left panel). Data are expressed as mean + SEM; n = 24. 5-LO product formation in granulocytes separated into trimesters (right panel). Data are means + SEM; n = 13 (2nd trimester (P2)), n = 11 (3rd trimester (P3)). (B) Granulocytes stimulated with A23187 plus AA (2.5 μM and 20 μM, respectively, 10 min, 37 °C, left panel). Data are means + SEM; n = 22. 5-LO product formation in granulocytes separated into trimesters (right panel). Data are means + SEM; n = 11 (P2), n = 11 (P3). (C) ^{3}[H]AA-release from granulocytes following stimulation with 1 μM A23187 for 5 min at 37 °C. Data are means + SEM; n = 2. (D) 5-LO product formation in homogenates of granulocytes from pregnant and non-pregnant donors stimulated with 1 mM ATP (30 s, 37 °C), 2 mM $CaCl_2$ and 20 μM AA (10 min, 37 °C). Data are means + SEM; n = 5. (E) Analysis of protein amount of 5-LO enzyme by immunodetection. One representative of 7 experiments is shown. Data passed normality Kolmogorov-Smirnov test;*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; pregnant vs. non-pregnant, paired t-test.

Elevated levels of ROS and related lipid hydroperoxides are known to favor the cellular activation of 5-LO (Rådmark et al., 2007). This, together with the observation that during pregnancy a lower ROS formation capacity was observed led to the hypothesis that lower LT synthesis might be originated in lower amounts of ROS formed (Kindzelskii et al., 2002, 2004). However, as shown in (Fig. 4.6A, left panel) an increase in ROS mimicked by addition of 13-HPODE did not increase LT formation neither in cells from non-pregnant nor from pregnant donors. Also, addition of DPI, an inhibitor of the NADPH oxidase did not interfere with 5-LO product formation (Fig. 4.6A, right panel). Note, that as already reported by others (Kindzelskii et al., 2002) lower ROS forming capacity in granulocytes from pregnant donors was observed after stimulation with ionomycin and PMA (Fig. 4.6B).

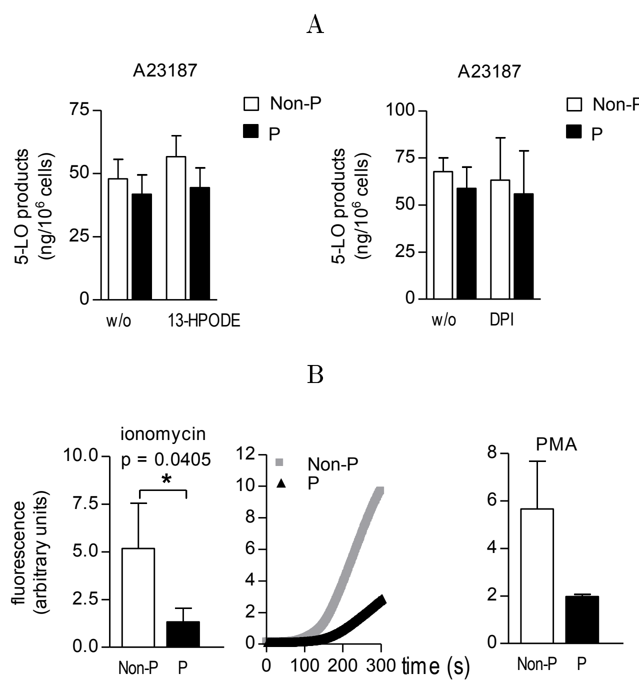

Figure 4.6: Impact of ROS formation in granulocytes on LT formation during pregnancy. (A) Granulocytes from pregnant (P) and non-pregnant (Non-P) donors were preincubated with 13-HPODE (left panel) or 10 μM DPI (right panel) for 3 min at 37 °C followed by stimulation with 2.5 μM A23187. Data are expressed as mean + SEM; n = 8 (left panel), n = 3 (right panel). (B) Granulocytes were stimulated with ionomycin (1 μM, left panel) or PMA (0.1 μM, right panel) and followed by fluorescence measurements of the ROS indicating dye DCF-DA. Data are expressed as mean + SEM; n = 3. *, $p < 0.05$; pregnant vs. non-pregnant, paired t-test of log transformed data. One representative experiment of at least 3 experiments (ionomycin stimulation) is shown (middle panel).

Stimulation of granulocytes with A23187 revealed also lower 12-H(p)ETE formation in granulocytes from pregnant donors (Fig. 4.7A, left panel). As also observed for 5-LO product formation, this difference disappeared by addition of substrate AA (Fig. 4.7A, right panel). Interestingly, 15-H(p)ETE formation was significantly lower in granulocyte fractions isolated from pregnant compared to non-pregnant donors (72%; 95% CI: 56-88%; Fig. 25B, left panel) after stimulation with A23187 plus AA. A significant linear correlation between the ratio of eosinophils in the granulocyte population and the amount of 15-H(p)ETE formed ($r^2 = 0.3$) (Fig. 4.7B, right panel) could be found.

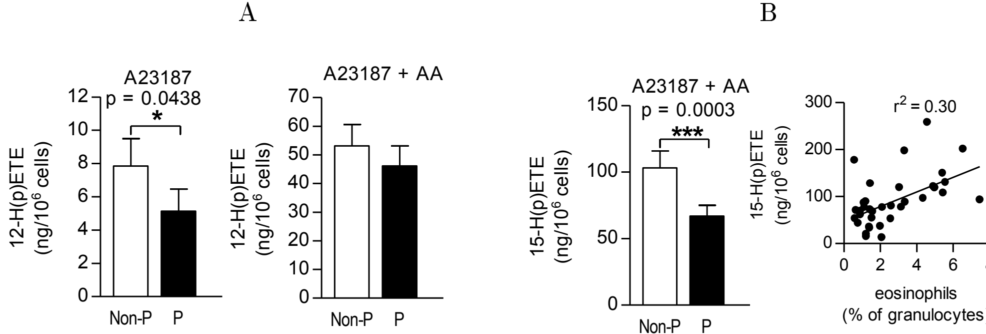

Figure 4.7: 12-H(p)ETE and 15-H(p)ETE formation in granulocytes from pregnant and non-pregnant females. (A) 12-H(p)ETE formation in granulocytes stimulated with 2.5 μM A23187 (left panel) or A23187 plus AA (2.5 μM and 20 μM, respectively, 10 min, 37 °C, right panel), n = 16 (A23187), n = 22 (A23187 plus AA). (B) 15-H(p)ETE formation in granulocytes stimulated with A23187 plus AA (2.5 μM and 20 μM, respectively, 10 min, 37 °C, left panel), n = 22. Data are presented as means + SEM. Data passed normality Kolmogorov-Smirnov test; *p $<$ 0.05; ***, p $<$ 0.001; pregnant vs. non-pregnant, paired t-test. (B, right panel) Linear regression of 15-H(p)ETE formation from granulocytes vs. ratio of eosinophils in granulocyte fractions.

4.1.4 Leukotriene formation in isolated PMBC

Next, the capacity for LT formation in isolated PBMC was analyzed. Within this fraction monocytes are mainly responsible for LT synthesis, while T-lymphocytes do not synthesize LTs at all and B-lymphocytes only possess low LT forming capacity under certain conditions (Werz, 2002). After stimulation of PMBC with A23187 higher LT levels in cells isolated from pregnant compared to non-pregnant donors were observed (about 1.7 fold, 95% CI: 1.1-2.3) contrasting the results observed for granulocytes (Fig. 4.8A, left panel). After providing exogenous AA, this difference was almost abolished (about 1.2 fold pregnant vs. non-pregnant donors, 95% CI: 0.9-1.5; Fig. 4.8A, middle panel). Interestingly, in homogenates of PBMC derived from pregnant females, the capacity to form LTs was higher compared to PBMC homogenates from non-pregnant donors (about 1.5 fold, 95% CI: 1.3-1.7; Fig. 4.8A, right panel). Considering the high numbers of monocytes in the blood of pregnant females (Fig. 4.4 and Fig. 4.8B, left panel) the idea is strengthened that higher LT synthesis in PBMCs from pregnant donors is related to the higher ratio of monocytes present in the PBMC preparations (1.4 fold, 95% CI: 1.2-1.6). After normalization of LT levels on the ratio of monocytes in the PBMC fraction, similar capacities to form LTs were evident in both samples from pregnant and non-pregnant females (Fig. 4.8B, right panel).
After stimulation of isolated PBMC with A23187 a trend towards higher 12-H(p)ETE formation was observed (Fig. 4.9A), which disappeared after treating cells with A23187 plus AA and was not evident in PBMC homogenates. No differences were observed for 15-H(p)ETE formation (Fig. 4.9B).

4.1.5 Impact of plasma from pregnant and non-pregnant women on LT formation in granulocytes and monocytes

During pregnancy various factors in the plasma change (Carlin and Alfirevic, 2008). In order to assess the effect of plasma on 5-LO product formation, three different preparations of human recombinant 5-LO enzyme (with different activities: a > b >

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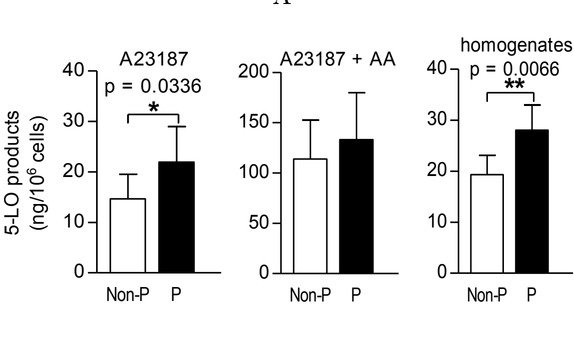

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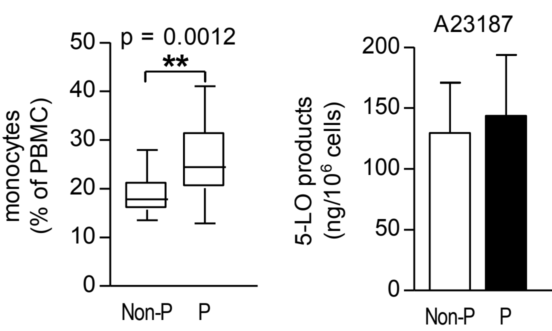
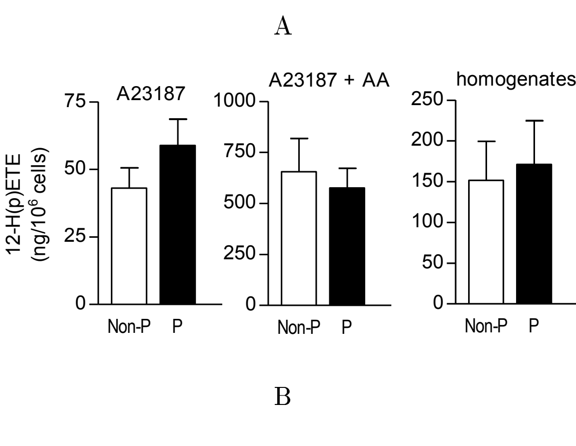


Figure 4.8: 5-LO activity is higher in PBMC isolated from blood of pregnant compared to non-pregnant donors. (A) 5-LO activity induced by A23187 (2.5 μM, 10 min, 37 °C, left panel). 5-LO activity induced by A23187 plus AA (2.5 μM and 20 μM, respectively, 10 min, 37 °C, middle panel) and in PBMC homogenates (right panel) stimulated with 1 mM ATP (30 s, 37 °C), 2 mM $CaCl_2$ and 20 μM AA (10 min, 37 °C) from pregnant (P) and non-pregnant (Non-P) donors. Data are expressed as mean + SEM; n = 11 (A23187), n = 11 (A23187 plus AA), n = 4 (homogenates). (B) Monocyte cell counts in percentage of PBMC cell count, n = 19 (left panel). 5-LO product formation normalized per 10^6 monocytes present in PBMC fraction (right panel), n = 6. Data passed normality Kolmogorov-Smirnov test; *, $p < 0.05$; **, $p < 0.01$; pregnant vs. non-pregnant, paired t-test.

A



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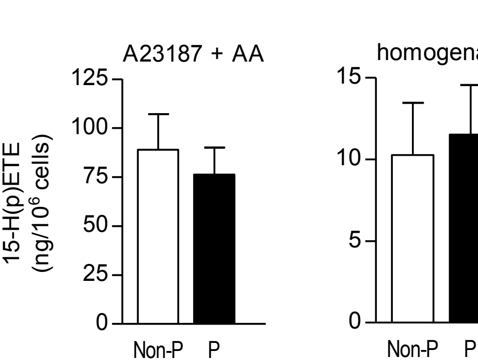

Figure 4.9: 12-H(p)ETE and 15-H(p)ETE formation in PBMC and PBMC homogenates from pregnant and non-pregnant females. (A) 12-H(p)ETE formation in PBMC induced by A23187 (10 min, 37 °C left panel), A23187 plus AA (2.5 μM and 20 μM, respectively, 10 min, 37 °C, middle panel) and in homogenates from PBMC of pregnant (P) and non-pregnant (Non-P) donors (1 mM ATP: 30 s, 37 °C, 2 mM $CaCl_2$ and 20 μM AA: 10 min, 37 °C, right panel). n = 11 (A23187), n = 11 (A23187 plus AA), n = 4 (homogenates). (B) 15-H(p)ETE formation in PBMC induced by A23187 plus AA (2.5 μM and 20 μM, respectively, 10 min, 37 °C, left panel) and in PBMC homogenates (1 mM ATP: 30 s, 37 °C, 2 mM $CaCl_2$ and 20 μM AA: 10 min, 37 °C, right panel). n = 11 (A23187 plus AA), n = 4 (homogenates). Data are means + SEM and passed normality Kolmogorov-Smirnov test.

c) were resuspended in plasma from pregnant or non-pregnant females. Interestingly, plasma from pregnant women showed a tendency to increase 5-LO activity in the cell-free assay (Fig. 4.10).

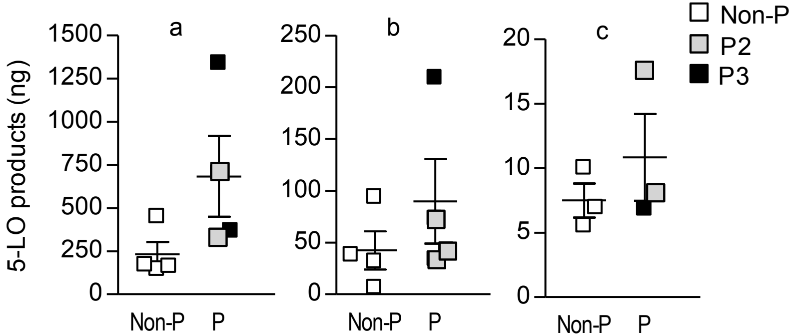

Figure 4.10: Effect of plasma from pregnant women on LT formation from purified 5-LO. Effect of plasma from pregnant women on 5-LO product formation by 3 different preparations of human recombinant 5-LO (enzymatic activity: a > b > c). Isolated 5-LO was resuspended in plasma from non-pregnant (Non-P) or pregnant (P) women for 5 min at 37 °C, and then stimulated with 1 mM ATP, 1 mM $CaCl_2$ (30 s, 37 °C) and 100 μM AA (10 min, 37 °C). Data are shown as dot plots. Results of plasma from different trimesters of pregnancy are shown (P2, 2nd; P3, 3rd trimester); lines represent mean ± SEM.

In order to evaluate if the plasma from pregnant females may also influence LT formation on the cellular level, corresponding granulocytes and monocytes were isolated from buffy coats derived from non-pregnant females. The cells were preincubated in the plasma for 5 min at 37 °C followed by stimulation of cells with A23187. Intriguingly, granulocytes resuspended in plasma from pregnant women showed higher LT formation capacity (about 1.5 fold, 95% CI: 1.1-1.8) than granulocytes resuspended in plasma from non-pregnant females (Fig. 4.11A, left panel). This up-regulation was higher for the plasma derived from pregnant females in the 2nd than in the 3rd trimester (1.6 fold, 95% CI: 1.8-2.1 and 1.3 fold, 95% CI: 0.66-1, for 2nd and 3rd trimester, respectively; Fig. 4.11A, right panel).

Incubation of monocytes in plasma and stimulation with A23187 did not show the same pattern as for granulocytes. Only a modest up-regulation was evident (Fig. 4.11B, left panel). However, dividing the groups into the trimesters revealed significantly higher LT formation by monocytes preincubated in plasma from pregnant women in the 2nd trimester compared to corresponding controls (1.8 fold, 95% CI: 0.8-2.7; Fig. 4.11B, right panel). After stimulation with A23187 plus AA, no difference was observed for granulocytes (Fig. 4.11C, left panel) as well as for monocytes (Fig. 4.11C, right panel) resuspended in the diverse plasma. In addition, this was also independent of the trimester (not shown). Furthermore, in two cases, granulocytes directly isolated from the blood of pregnant women were incubated in plasma from non-pregnant women and vice-versa. As shown in Fig. 4.11D cells from pregnant females resuspended in autologous plasma led to higher LT formation compared to cells in heterologous plasma from non-pregnant females. Note that for the combination of cells from non-pregnant females and plasma it is not possible to statistically analyze the data since the sample number is too small.

Next, the influence of plasma isolated from blood of pregnant females on the release of AA by $cPLA_2$ in granulocytes (buffy coat derived, non-pregnant female) was analyzed. For this purpose granulocytes were labeled with [^{3}H]AA followed by preincubation with plasma and stimulation with A23187. As shown in Fig. 4.12, plasma from pregnant females did not lead to significant higher release of [^{3}H]AA compared to plasma from non-pregnant donors.

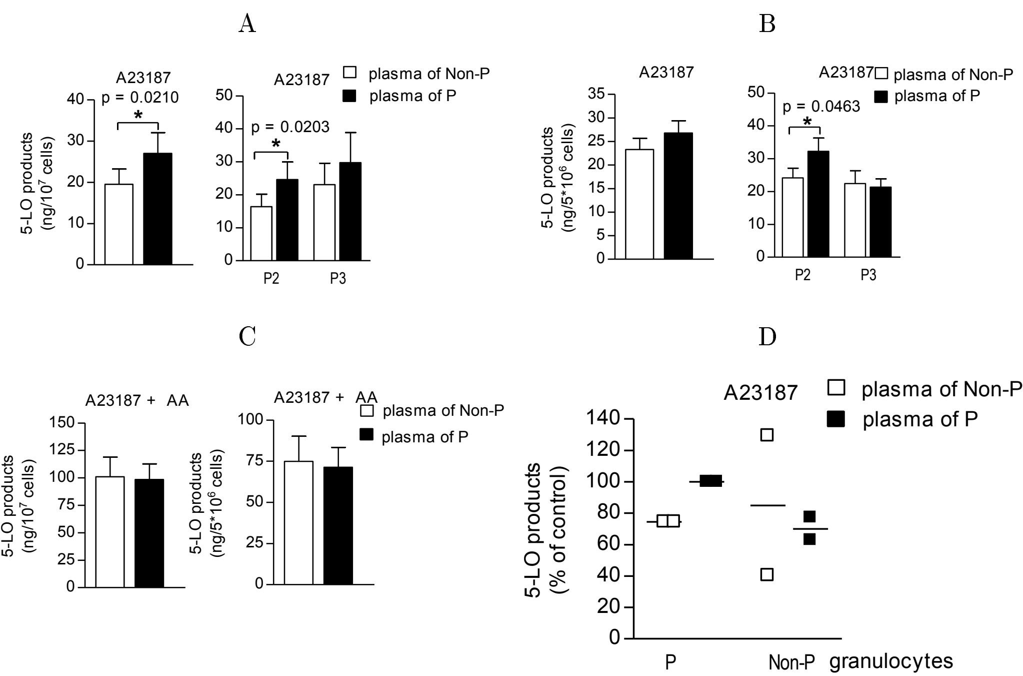

Figure 4.11: Effect of plasma from pregnant (P) and non-pregnant (Non-P) donors on 5-LO product formation in granulocytes and monocytes from non-pregnant females. (A) 5-LO product formation by granulocytes from Non-P females stimulated with A23187 (30 μM, 10 min, 37 °C) after preincubation with plasma from P and Non-P donors for 5 min, 37 °C (left panel). Data are means + SEM; n = 19 (A23187). Separation into trimesters (right panel). Data are means + SEM; n = 10 (2nd trimester (P2)), n = 9 (3rd trimester (P3)). (B) 5-LO product formation by monocytes from Non-P females stimulated as in (A). Data are expressed as mean + SEM; n = 20. Separation into trimesters (right panel). Data are expressed as + SEM; n = 10 (P2), n = 10 (P3). (C) 5-LO product formation by granulocytes (left panel) and monocytes (right panel) from Non-P females stimulated with A23187 plus AA (30 μM and 100 μM, respectively, 10 min, 37 °C) after preincubation with plasma from P and Non-P donors for 5 min, 37 °C. Data are means + SEM; n = 18 (granulocytes), n = 19 (monocytes). (D) Granulocytes resuspended in plasma and stimulated with A23187. Data are normalized on cells and plasma from P donors. Data are means + SEM; n = 2. Data passed normality Kolmogorov-Smirnov test; *, $p < 0.05$; P vs. Non-P, paired t-test.

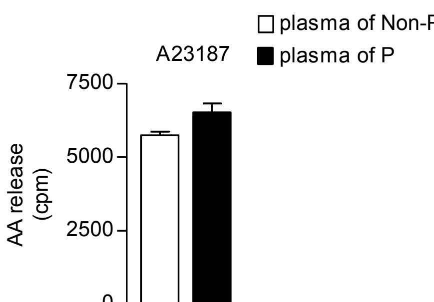

Figure 4.12: Effect of plasma isolated from blood from pregnant and non-pregnant donors on AA release from granulocytes. ^{3}[H]AA release by granulocytes from non-pregnant females stimulated with A23187 (30 μM, 5 min, 37 °C) after preincubation with plasma from pregnant (P) and non-pregnant (Non-P) donors for 5 min, 37 °C (left panel). Data are expressed as mean + SEM; n = 3.

4.1.6 Impact of plasma from pregnant donors on ROS formation in granulocytes

As already reported elsewhere serum or plasma from pregnant women induces ROS formation in neutrophils (Crocker et al., 1999). Here, we show data using the ROS-sensitive dye DCF-DA to monitor intracellular ROS formation in neutrophils, while others used a chemiluminescence method. As shown in Fig. 4.13A (left panel),

plasma from pregnant females did not potentiate fMLP-induced intracellular ROS formation in granulocytes compared to non-pregnant females after short time incubation (6 min). However, after 100 min ROS formation in granulocytes was higher in samples incubated with plasma of pregnant compared to plasma of non-pregnant females (1.2 fold, 95% CI: 1.2-1.3, Fig. 4.13A, right panel). Also the plasma itself caused a fluorescence signal which was higher for samples from pregnant donors after 100 min incubation (2.1 fold, 95% CI: 1.7-2.5) (Fig. 4.13B). Heat inactivation of plasma (60 min, 56 °C) increased the upregulating effect of plasma from pregnant females (Fig. 4.13C).

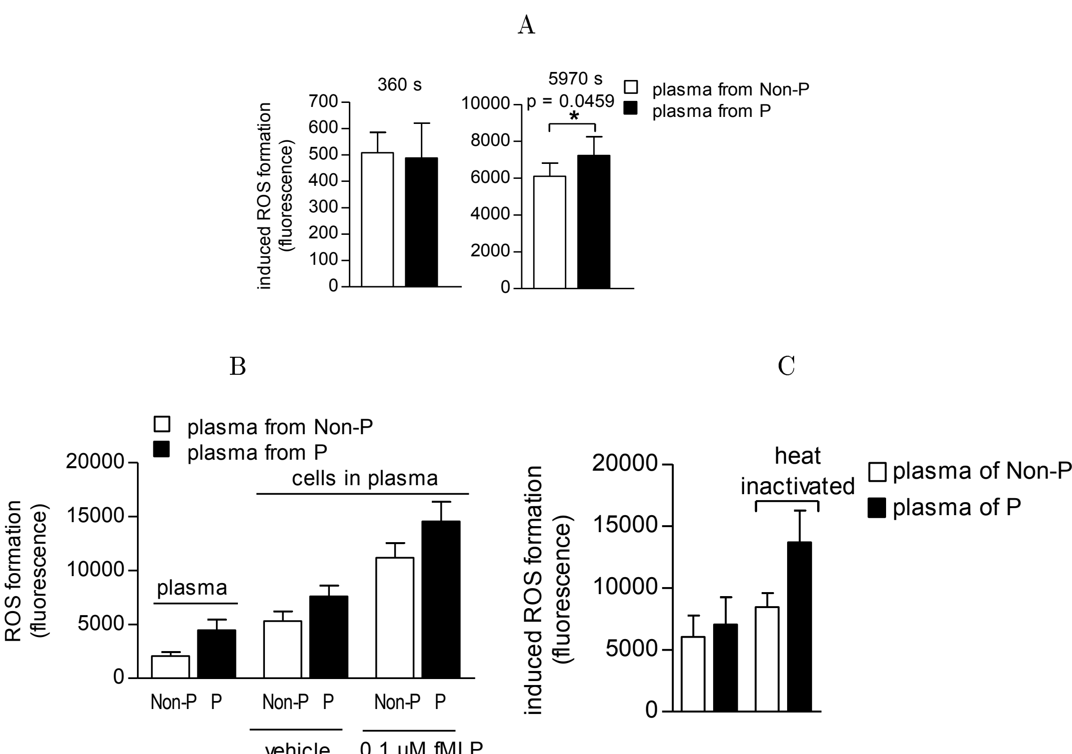

Figure 4.13: Influence of plasma on ROS formation. Female neutrophils 5×10^5/well in PG buffer were incubated with 1 μg/ml DCF-DA for 5 min at 37 °C. After addition of plasma from pregnant (P) and non-pregnant (Non-P) donors (5% final concentration) for 5 min at 37 °C, cells were stimulated with 0.1 μM fMLP (100 min, 37 °C). The fluorescence signal was detected. Data after 360 s and 5970 s are shown as induced fluorescence. Data passed normality Kolmogorov-Smirnov test; *, $p < 0.05$; P vs. Non-P, paired t-test. (B) ROS formation after 5970 s. Data are expressed as mean + SEM; n = 11. (C) Influence of heat-inactivation of plasma on ROS formation after 5970 s. Data are mean + SEM; n = 4.

4.1.7 Influence of female sex hormones on leukotriene formation

During pregnancy female sex hormones increase to a high extent (O'Leary et al., 1991). Progesterone and estradiol levels were higher in samples from pregnant compared to non-pregnant females by 44.2- (95% CI: 27.1-237.1) and 109.9-fold (95% CI: 64.0-155.8), respectively (Table 4.2). While the testosterone concentration was only 1.6-fold (95% CI: 1.3-2.0) higher in samples from pregnant donors.

Tabelle 4.2: Plasma concentrations of sex hormones. Data are presented as median and range; n = 20.

	pregnant	non-pregnant
progesterone (nM)	312.7 (88.9-1,454)	8.10 (1.0-68.0)
estradiol (pM)	34,495 (3,670-98,245)	378.5 (71.0-1,809)
testosterone (nM)	2.85 (1.8-6.0)	1.95 (0.9-3.3)

Therefore, it seemed reasonable to investigate if 5-LO product formation is influenced by female sex hormones. Testosterone inhibits 5-LO product formation in both monocytes and neutrophils via a direct non-genomic mechanism as reported, which causes a differential LT formation capacity in blood derived males and females (Pergola et al., 2008, 2011). As Fig. 4.14 shows, female sex hormones (estradiol and progesterone) did not influence LT synthesis capacity in monocytes after 90 min incubation whereas 5-*alpha*-dihydrotestosterone (5α-DHT), the active metabolite of testosterone, inhibited 5-LO product formation in female monocytes. In parallel, also 12-H(p)ETE and 12-HHT were analyzed. For all products lower amounts were produced in monocytes incubated with sex hormones. The strongest effects were observed for 5α-DHT. Since lower capacity to form LO derived products might originate in lower viability of the cells MTT assays were performed after 90 min preincubation with the hormones. As shown (Fig. 4.14), the viability of the cells was not influenced by the sex hormones.

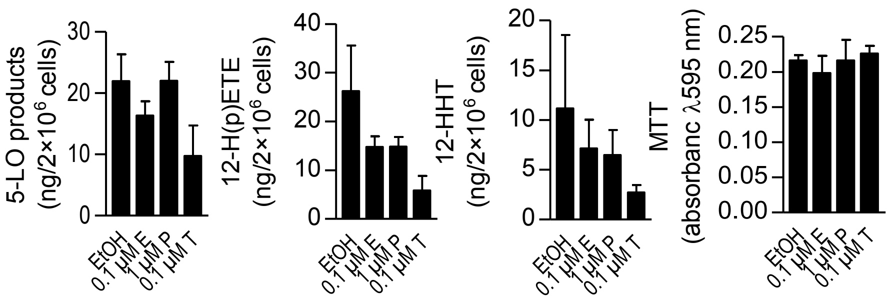

Figure 4.14: Influence of sex hormones on diverse LO and COX-1 product formation in monocytes. Female monocytes (10^6 cells/ml RPMI 1640, 2% stripped human serum were preincubated for 90 min at 37 °C, 6% CO_2 with hormones or vehicle (0.05% ethanol). Product formation was induced by 5 μM A23187 (10 min, 37 °C). Data are means+ SEM; n = 3. E = estradiol, P = progesterone, T = 5α-DHT. MTT analysis of monocytes incubated for 90 min at 37 °C and 6% CO_2 with hormones or vehicle (0.05% ethanol). MTT was added for 4 h at 37 °C followed by cell lysis. Absorbance was measured at 595 nm (Victor[3] plate reader, Perkin Elmer, Rodgau-Jügesheim, Germany). Data are means + SEM; n = 3

4.1.8 Influence of plasma components on leukotriene formation

During pregnancy several proteins and also glycoproteins are upregulated in the plasma (Carlin and Alfirevic, 2008). To this aim, the influence of leptin and macrophage inhibitory factor (MIF) on LT formation was assayed. Both modulators are related to inflammatory diseases and levels are altered during pregnancy (Hardie et al., 1997; Sivan et al., 1998; Hristoskova et al., 2006). In addition, the influence of the pregnancy-associated hormone human chorionic gonadotropin (HCG) on LT formation in neutrophils was analyzed. HCG is a modulator of the immune tolerance during pregnancy (Schumacher et al., 2013). However, as shown in Fig. 4.15 no factor (in physiological concentration) analyzed upregulated LT formation in the different assays used.

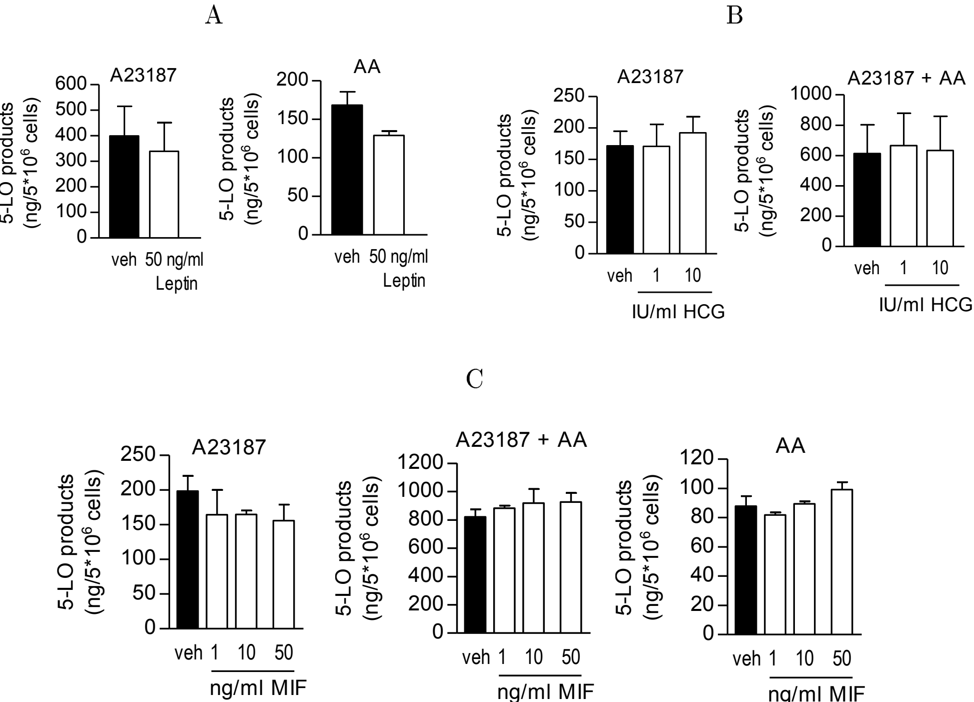

Figure 4.15: Effect of pro-inflammatory proteins on LT formation in neutrophils. (A) Female neutrophils were preincubated for 30 min at 37 °C with 50 ng/ml leptin followed by stimulation with 2.5 μM A23187 (left panel) or 20 μM AA (right panel) for 10 min at 37 °C. Data are means + SEM; n = 3. (B) Female neutrophils were preincubated with HCG for 5 min at 37 °C followed by stimulation with 2.5 μM A23187 (left panel) or 2.5 μM A23187 plus 20 μM AA (right panel) for 10 min at 37 °C. Data are expressed as mean + SEM; n = 3. (C) Female neutrophils were preincubated with MIF (ng/ml) for 5 min at 37 °C followed by stimulation with 2.5 μM A23187 (left panel), 2.5 μM A23187 plus 20 μM AA (middle panel), 20 μM AA (right panel) for 10 min at 37 °C. Data are means + SEM; n = 2.

4.1.9 Summary: The influence of pregnancy on LO derived product formation

In this chapter it is demonstrated that upon stimulation LT levels are higher in peripheral blood derived from pregnant donors compared to non-pregnant females. This was evident for both 2nd and 3rd trimester. Elucidation of the origin for this up-regulation led to the following observations: (I) increased number and proportions of cells in blood responsible for the synthesis of LTs in blood from pregnant females ("plus effect"); (II) impaired LT formation capacity of isolated granulocytes from pregnant females ("minus effect"); (III) higher capability to form LTs for cells (granulocytes and also partially monocytes) that were resuspended in plasma derived from pregnant donors ("plus effect"). As illustrate in Fig. 4.16, the increased capacity of blood from pregnant woman to form LTs is driven by factors interacting via synergism or into contrary directions.

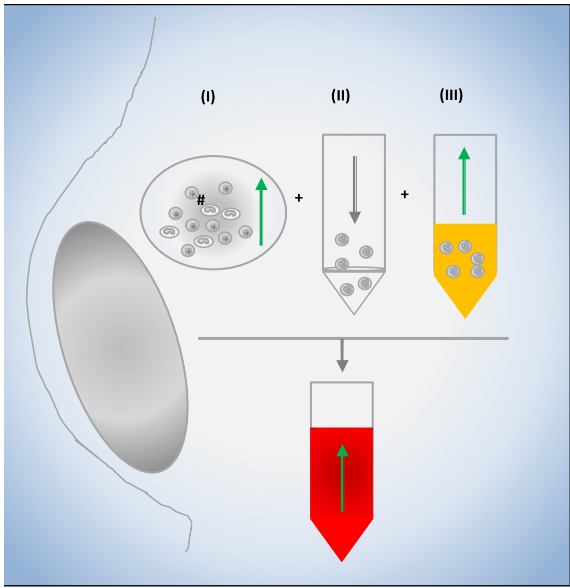

Figure 4.16: Summary: High leukotriene formation in peripheral blood during pregnancy.

4.2 Impact of menstrual cycle on LO and COX-1 product formation in blood

During female menstrual cycle hormonal changes occur which have major influences on the immune system as well as on the course of autoimmune diseases (Whitacre, 2001). Eicosanoids are reported to play an important role in the reproductive tract during female menstrual cycle (Sales and Jabbour, 2003). Besides, LTs are known to be increased in serum of female patients suffering from dysmenorrhea which do not respond to anti-prostaglandin therapy (Abu and Konje, 2000). Furthermore, premenstrual asthma has been described which can be successfully treated with CysLT antagonists (Nakasato et al., 1999). However, some reports are disputing this (Pereira-Vega et al., 2012). Samples of the non-pregnant controls from the pregnancy study were subdivided between donors in the follicular phase (cycle day 1 to 13) and the luteal phase (cycle day 14 to 30). Female sex hormone concentrations in plasma were monitored to further control the indication of the cycle day by the donors. As shown in Fig. 4.17A, LT formation stimulated with fMLP was higher in blood from women in the luteal phase versus the follicular phase. After stimulation with A23187 the difference was not significant and after supply of substrate AA this difference was even more reduced (Fig. 4.17A). In contrast, eicosanoids like 12-H(p)ETE (Fig. 4.17B) and prostanoid 12-HHT (Fig. 4.17C), that in blood are mainly derived from platelets, were higher in samples from donors in the follicular phase regardless the stimulus used. 15-H(p)ETE formation was not different between the two phases (stimulation: A23187 plus AA) (Fig. 4.17D).
To further elucidate this differential pattern (downregulation of 5-LO-derived LTs and upregulation of 12-HHT and 12-H(p)ETE during follicular phase; during luteal phase vice versa), cell numbers, 5-LO activity of isolated cells and impact of plasma on eicosanoid formation were analyzed. Immune cell and erythrocyte counts were not different between the two phases (Fig. 4.18). Interestingly, platelet counts were higher in the follicular phase (Fig. 4.18).
However, 12-HHT formed in blood did not correlate significantly with the number of platelets present in the blood (Fig. 4.19).

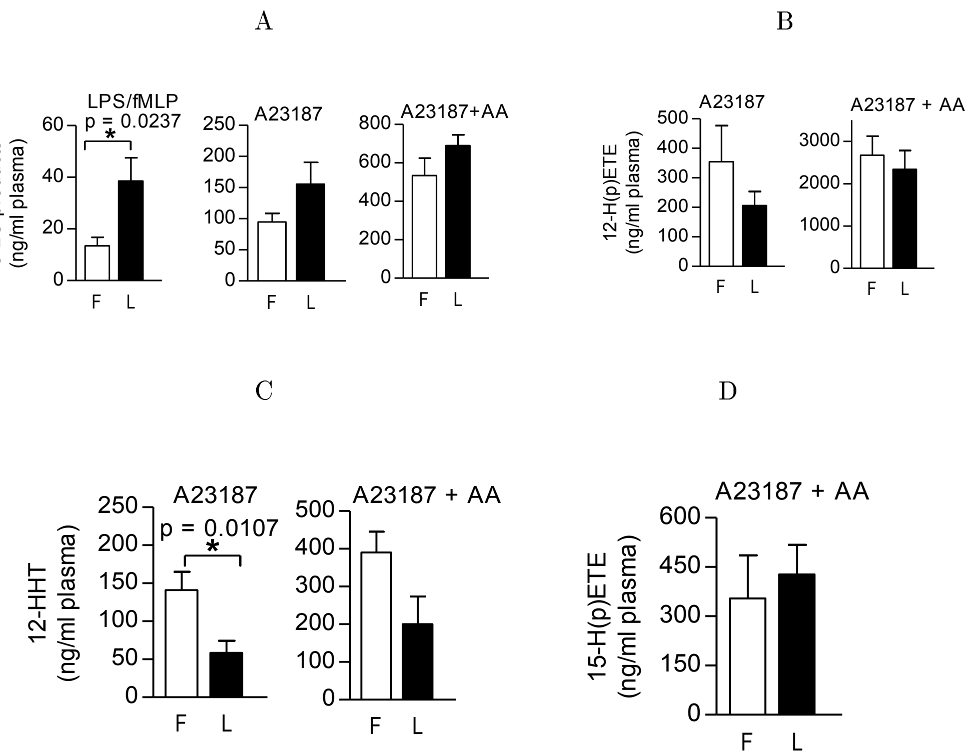

Figure 4.17: Eicosanoid formation in blood of females in the follicular (F) or the luteal (L) phase. (A) 5-LO product formation in blood from female donors induced by fMLP (1 μM, 15 min, 37 °C, left panel) after priming with LPS (1 μg/ml, 30 min, 37 °C), with A23187 (30 μM, middle panel) or A23187 plus AA (30 μM and 100 μM, respectively, right panel) for 10 min, at 37 °C. Data are means + SEM; fMLP: n = 8 (follicular phase (F)), n = 10 (luteal phase (L)); A23187: n = 10 (F), n = 12 (L); A23187 plus AA: n = 9 (F), n = 10 (L). *, $p < 0.05$; F vs. L; unpaired t-test with Welch's correction. (B) 12-H(p)ETE formation induced by A23187 (30 μM, left panel) or A23187 plus AA (30 μM and 100 μM, respectively, right panel) for 10 min at 37 °C. Data are means + SEM; A23187: n = 10 (F), n = 12 (L), A23187 plus AA: n = 9 (F), n = 10 (L). (C) 12-HHT formation induced by A23187 (30 μM, left panel) or A23187 plus AA (30 μM and 100 μM, respectively, right panel) for 10 min at 37 °C. Data are means + SEM; A23187: n = 7 (F), n = 9 (L), A23187 plus AA: n = 7 (F), n = 7 (L). *, $p < 0.05$; F vs. L; unpaired t-test. (D) 15-H(p)ETE formation induced by A23187 plus AA (30 μM and 100 μM, respectively, 10 min, 37 °C). Data are means + SEM; A23187 plus AA: n = 9 (F), n = 10 (L).

LT formation in isolated granulocytes from females at different time points of the menstrual cycle was compared and no differences between luteal and follicular phase were observed after stimulation with A23187 (Fig. 4.20A). Also, no effect of plasma on LT formation either from neutrophils or from monocytes resuspended in plasma (Fig. 4.20B) could be detected.
In summary, 5-LO product formation in stimulated blood was higher while 12-HHT formation was lower during luteal phase compared to follicular phase (Fig. 4.21). Neither the plasma, the numbers of cells or product formation capacity of the respective cells were responsible for this observation.

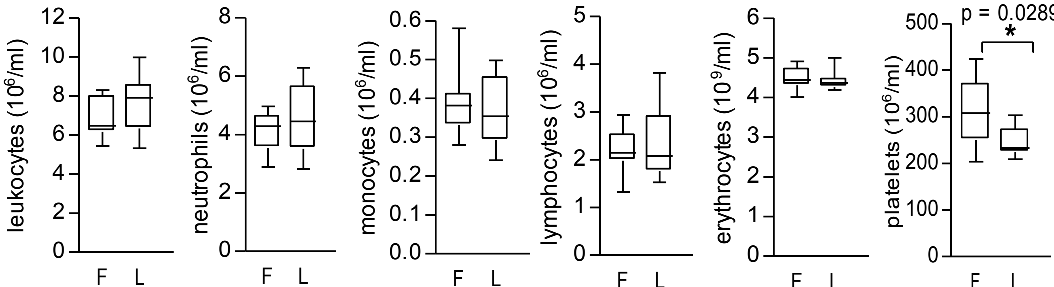

Figure 4.18: Blood cell counts of females in the follicular (F) or the luteal (L) phase. Data are expressed as 25th, 50th and 75th percentiles and the range; n = 9 (follicular phase (F)), n = 9 (luteal phase (L)). *, $p < 0.05$; F vs. L unpaired t-test with Welch's correction.

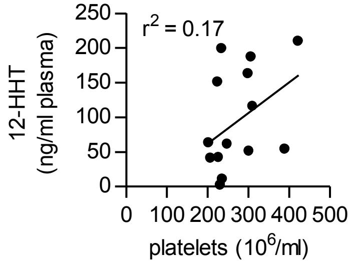

Figure 4.19: Correlation of 12-HHT formation in blood and platelet cell count. Linear regression of 12-HHT formation in blood after stimulation with 30 μM A23187 vs. number of platelets. n = 14.

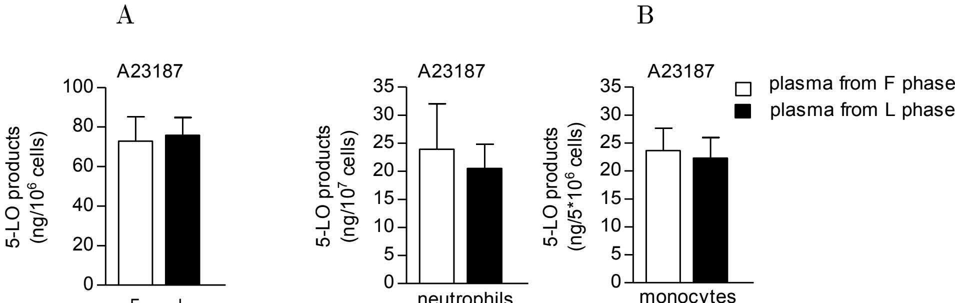

Figure 4.20: Influence of female cycle on LT formation in isolated granulocytes and the effect of plasma. (A) Female granulocytes isolated from blood were incubated with 2.5 μM A23187 for 10 min at 37 °C (left panel). Data are expressed as means + SEM; n = 10 (follicular phase (F)), n = 9 (luteal phase (L)). (B) Female neutrophils (left panel) or monocytes (right panel) isolated from buffy coats were resuspended in plasma from female donors during F and L phase of the cycle, preincubated for 5 min at 37 °C and stimulated with 30 μM A23187 for 10 min at 37 °C (left panel). Data are expressed as means + SEM; neutrophils: n = 8 (F), n = 10 (L); monocytes: n = 8 (F), n = 10 (L).

4.3 Inhibition of 5-LO by embelin

Interestingly, some naturally derived compounds share the features of fatty acids and have been presented as modulators of the 5-LO pathway (Werz, 2007). The benzohydroquinone embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone) (Fig. 4.22) which is occurring in species of *Embelia ribes* (Myrsinaceae) incorporates a fatty acid-like structure. Several animal studies demonstrate anti-inflammatory activity of embelin *in vivo*, e.g. in colitis models (Kalyan Kumar et al., 2011; Thippeswamy et al., 2011),

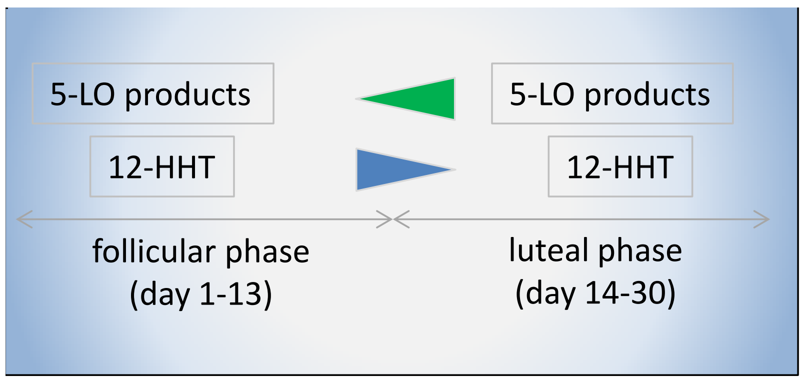

Figure 4.21: Summary of the investigations on the influence of female cycle on LO and COX-1 product formation.

models of skin inflammation (Kumar et al., 2011) and in carrageenan-induced paw edema (Mahendran et al., 2011b). Furthermore, marked anti-tumor activities were described (Dai et al., 2011, 2009). After embelin treatment several underlying targets for the pharmalogical effects *in vivo* were identified. Myeloperoxidase activity, expression of inducible nitric oxide synthase, NO formation, release of TNFα, IL-1β and IL-6 were lower in isolated colon tissue from dextrane sulfate sodium-induced colitis in mice (Kalyan Kumar et al., 2011) and TNFα levels were impaired in ear tissue in a model of skin inflammation (Kumar et al., 2011). The expression of the NFκB target genes COX-2, survivin, cyclin D1, and c-Myc was downregulated by embelin (Dai et al., 2009). Direct inhibitory effects of embelin on X-linked inhibitor of apoptosis protein (XIAP; IC_{50} = 4.1 μM) may explain its anti-tumor activity in XIAP overexpressing cancer cell lines (Nikolovska-Coleska et al., 2004) and $PPAR_\gamma$ signaling is seemingly involved in the inhibitory action of embelin on colon cancer cells (Dai et al., 2009). However, except for XIAPs, relevant direct molecular targets of embelin and concrete modes of action are still elusive. The study presented here was published in (Schaible et al., 2013b).

Figure 4.22: Chemical structure of embelin

4.3.1 Molecular characterization of the inhibition of 5-LO by embelin

Embelin was first tested in an isolated enzyme model of partially purified 5-LO and the IC_{50} value was determined at 0.06 ± 0.01 μM (Fig. 4.23A). Interestingly, embelin inhibited 5-LO product formation in homogenates of human PMNL in a similar manner with an IC_{50} = 0.11 ± 0.01 μM (Fig. 4.23A). Embelin was superior over the only inhibitor that has been introduced onto the market so far – zileuton (Carter et al., 1991) (Fig. 4.23B, isolated 5-LO: IC_{50} = 0.6 ± 0.1 μM; homogenates: IC_{50} = 0.8 ± 0.2 μM). Furthermore, the activity of 12- and 15-LOs which are related to 5-LO were not impaired by embelin up to 1 μM in homogenates from PMNL (Fig. 4.23C).

Since some inhibitors are reported to irreversibly bind to 5-LO (Hornig et al., 2012),

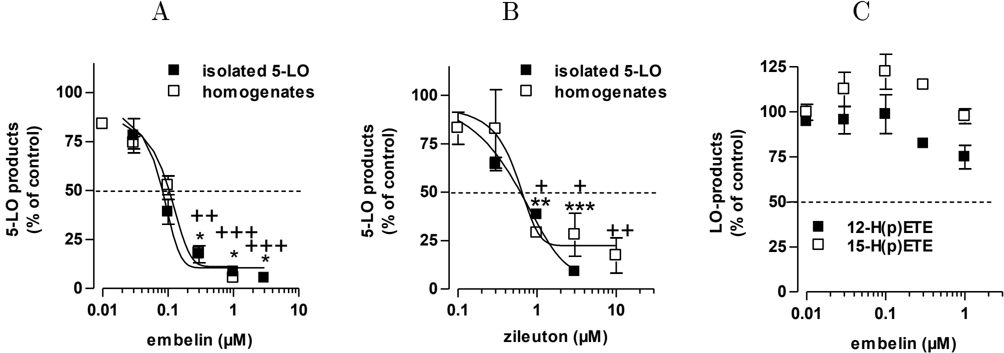

Figure 4.23: Inhibition of 5-LO by embelin in cell-free assays. Partially purified recombinant 5-LO[a] or homogenates of PMNL (corresponding to 5×10^6 cells/ml) were incubated with embelin (A), zileuton (B) or vehicle (DMSO, 0.1%) at 4 °C for 15 min. Samples were prewarmed for 30 s at 37 °C, 2 mM $CaCl_2$ and 20 μM AA were added (10 min, 37 °C). Data are expressed as percentage of control (100%), means ± SEM, n = 3-6. Purified 5-LO: *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ versus 100% control; neutrophil homogenates: +, $p < 0.05$, ++, $p < 0.01$, +++, $p < 0.001$ versus 100% control. (C) Effect of embelin on 12-H(p)ETE and 15-H(p)ETE formation in PMNL homogenates stimulated as described in (A). [a]The assay was performed by H. Traber (University of Jena).

the reversibility of the inhibition was tested with a wash-out experiment. Partially purified 5-LO was incubated with 0.3 μM embelin to reach complete inhibition. One aliquot was diluted 10-fold (0.03 μM embelin). The other aliquot remained untreated. After stimulation of the preparations, the amount of LTs formed was analyzed. As shown in Fig. 4.24A, the inhibition was reversible. Wiegard et al. argue that highly active inhibitors often behave as nuisance inhibitors which precipitate the enzyme protein by forming colloid-like aggregates (Wiegard et al., 2012). This unspecific inhibition can be prevented by the addition of non-ionic detergents (such as triton X-100), which prohibit the formation of such aggregates. After addition of 0.01 or 0.1% triton X-100 to the enzyme preparations the IC_{50} value shifted from 0.07 ± 0.03 μM to 0.25 ± 0.04 μM (Fig. 4.24B). But embelin still inhibited 5-LO product formation in a concentration dependent manner. Therefore, one can exclude that the formation of aggregates is the main mechanism responsible for the inhibition of 5-LO. Considering the fatty acid-like structure of embelin a competitive mechanism might be possible. However, the inhibitory potential of embelin was not influenced by varying concentrations of AA (5 to 80 μM) supplied to partially purified 5-LO. The IC_{50} values ranged from 0.09 to 0.4 μM. Interestingly, the highest IC_{50} value was observed for the lowest AA concentration tested (5 μM). However, an uncompetitive mechanism was not obvious (Fig. 4.24C).

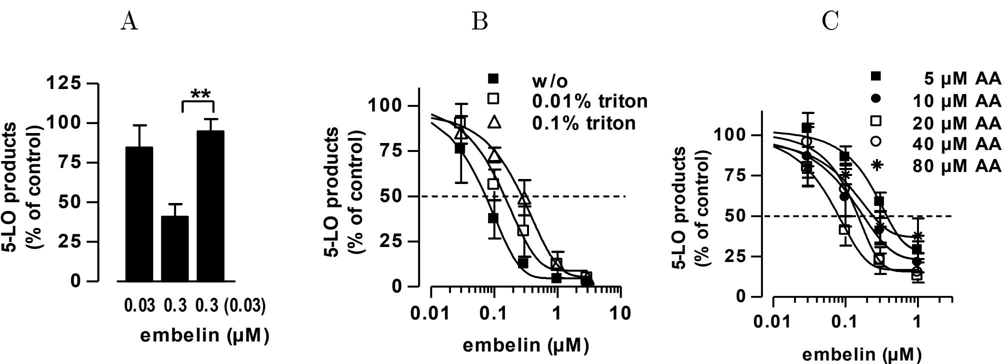

Figure 4.24: Characterisation of 5-LO inhibition by embelin. (A) Reversibility of 5-LO inhibition by embelin. Purified 5-LO was incubated with 0.03 μM or 0.3 μM embelin for 15 min at 4 °C. An aliquot of the 0.3 μM sample was diluted with assay buffer 10-fold ("0.3 (0.03)"); the other aliquot was not altered. Then, samples were prewarmed for 30 s at 37 °C and 20 μM AA and 2 mM $CaCl_2$ (10 min, 37 °C). **, p < 0.01 versus inhibition without dilution. (B) Purified 5-LO was incubated with embelin in the absence or presence of 0.01 or 0.1% triton-X 100 and 5-LO activity was determined as mentioned above. (C) Purified 5-LO was preincubated with embelin or vehicle (DMSO, 0.1%, 15 min, 4 °C) and 2 mM $CaCl_2$ plus the indicated concentrations of AA (10 min, 37 °C).[a] Data are expressed as percentage of control (100%), means ± SEM, n = 3-4. [a]Assay was done by H. Traber (University of Jena).

For 1,4-benzoquinones, it has been shown that they interfere with the redox cycle of 5-LO (Poeckel et al., 2006). This feature was investigated by the addition of 1 mM DTT to homogenates from PMNL in which glutathione-peroxidases are present. The need for glutathione-peroxidases for some inhibitors to be fully active has been described before (Werz et al., 1998). However, as shown in Fig. 4.25 (left panel), addition of the reducing agent DTT did not change the inhibitory potency of embelin. As reference inhibitor for this test system the 1,4 benzoquinone AA-861 (Yoshimoto et al., 1982) was used (Fig. 4.25, right panel), which behaved as expected.

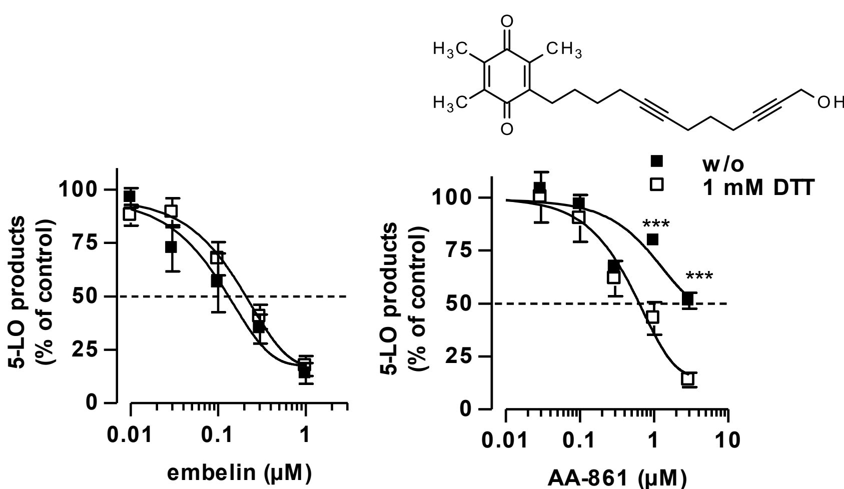

Figure 4.25: Effect of reducing conditions on the inhibitory capacity of embelin. PMNL homogenates were incubated with embelin (left panel), AA-861 (right panel) or vehicle (DMSO, 0.1%) for 15 min at 4 °C. 5 min prior to stimulation, 1 mM DTT was added as indicated. Samples were warmed up for 30 s at 37 °C and stimulated with 2 mM $CaCl_2$ and 20 μM AA for 10 min at 37 °C. Data are expressed as percentage of control (100%), means ± SEM, n = 3-4.

To further elucidate the mechanisms underlying the inhibition of 5-LO by embelin a docking simulation fitting of embelin to the binding pocket of the "stable" 5-LO crystal structure (Gilbert et al., 2011) was conducted in collaboration with Dr. Daniela Schuster (University of Innsbruck, Austria) and published in (Schaible et al., 2013b). In total six conceivable chemical structures of embelin (embelin, hydroembelin and four deprotonated forms of hydroembelin) were docked without constraints (Fig. 44). All six variants gave a similar dominant resulting pose. The hydrophobic n-undecanyl residue fills the hydrophobic channel running by the catalytic iron, where the oxygenation of AA would take place in the non-inhibited state. The hydroxylated quinone ring is coordinated by the three amino acids Gln-363, Gln-557, and Tyr-181, participating in hydrogen bonds. Two water molecules were observed to mediate binding of embelin. H2O855 mediates a hydrogen bridge between embelin and Thr-364 of the protein, while H2O802 is located between Asn-425 and the ligand.

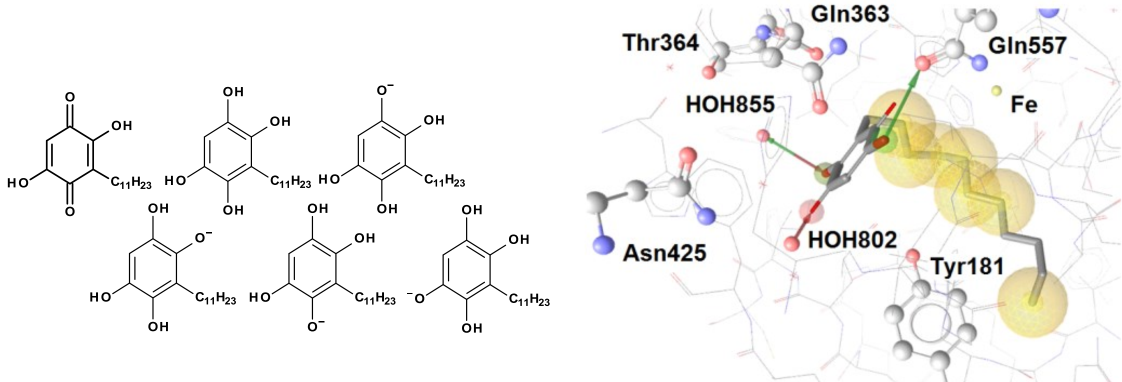

Figure 4.26: Molecular docking simulations of embelin into 5-LO. Chemical structural variants of embelin: Quinone state, hydroquinone state and different deprotonation states of the hydroquinone. An exemplary docking pose is shown. The n-undecanyl chain fills the hydrophobic substrate channel, running by the catalytic iron. The quinone ring is lodged between Gln-363, Gln-557, and Tyr-181, forming several stabilizing hydrogen bonds, with two cocrystallized water molecules within the binding pocket, H2O855 and H2O802, acting as mediators. Chemical interactions between embelin and the 5-LO binding site are color-coded: yellow sphere – hydrophobic interaction, red arrow – hydrogen bond acceptor, green arrow – hydrogen bond donor. The docking was performed by the group of Dr. Daniela Schuster, University of Innsbruck, Austria and published in (Schaible et al., 2013b).

Interestingly, Tyr-181 was postulated to be involved in the coordination of embelin at the catalytic domain. Together with Phe-177, Tyr-181 is discussed as a lid or a cover to close the entry side for the substrate AA to the catalytic center of 5-LO (Gilbert et al., 2011). In order to proof whether or not embelin mediates inhibition of 5-LO via binding to Tyr-181, the inhibitory capacity was tested at the 5-LO mutant Y181A in which Tyr-181 is replayced by alanine (established by Jana Gerstmeier, University of Jena). This mutant differs from wild-type 5-LO in several aspects: (I) optimal concentration of substrate AA, (II) ratio of the formation of LTB_4 isomers vs. 5-H(p)ETE and (III) influence of the presence of Ca^{2+}. Since the different preparations of the partially purified enzyme had varying activities the influence of different substrate concentrations were calculated as percentage of product formation of the stimulation with 20 μM AA. As shown in Fig. 4.27A, the amount of 5-LO products formed by the 5-LO mutant Y181A was affected by different concentrations of AA, with 10 μM AA leading to higher amounts of products formed than at 20 μM or

40 μM. For wild-type 5-LO, 20 μM AA was optimal. For both wild-type as well as mutant Y181A, 40 μM AA led to lower product formation (Fig. 4.27A). The ratio of 5-H(p)ETE and LTB_4 isomer formation increased with the AA concentration tested (10, 20, 40 μM). Interestingly, this increase was more pronounced for the mutant than for the wild-type 5-LO (Fig. 4.27B). From 10 μM to 40 μM AA the increase was 3.8-fold for the mutant and 1.9-fold for wild-type 5-LO. Whereas the activity of the Y181A mutant was not influenced by the presence or absence of 1 mM $CaCl_2$, the wild-type showed a tendency for higher product formation at the absence of Ca^{2+} ions (Fig. 4.27C) after stimulation with 20 μM AA. The capacity of definite amounts of enzyme protein to form products was not investigated in this thesis and is part of future analysis in the work group. Only a rough estimation was done which brought the hypothesis that the mutant was more active than the wild-type. This phenomenon was also reported by Dr. Bettina Hofmann, University of Frankfurt (personal communication).

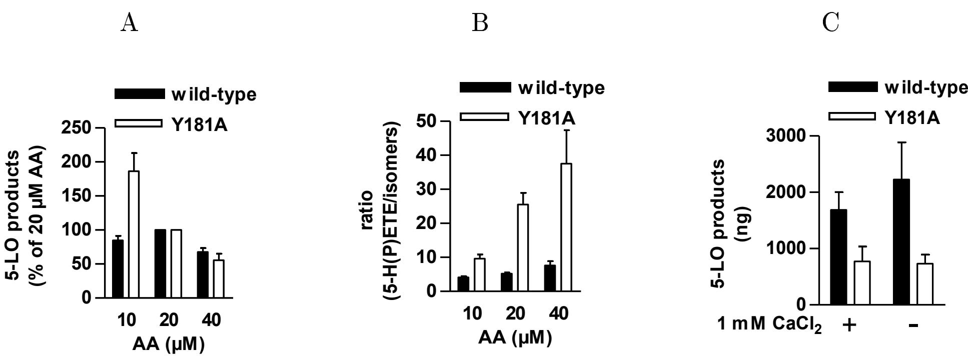

Figure 4.27: Characterisation of the 5-LO mutant Y181A. Preparations of partially purified wild-type and mutant Y181A 5-LO were prewarmed for 30 s at 37 °C after addition of 1 mM ATP and stimulated with AA and Ca^{2+} for 10 min at 37 °C as indicated. Data are expressed as mean + SEM. n = 3.

Interestingly, no differences were observed for the inhibitory capacity of embelin to inhibit wild-type 5-LO or the Y181A mutant regardless of the stimulation conditions used (Fig. 4.28). No difference was observed for the absence of $CaCl_2$ or varying concentrations of AA for both wild-type and Y181A mutant (Fig. 4.28B and C).

4.3.2 Efficiency of embelin to inhibit cellular leukotriene formation

Next, cellular assays were applied to study to the potency of embelin in a more physiologic environment. Stimulation of human PMNL or monocytes with Ca^{2+}-ionophore A23187 or with pathophysiologically relevant agonists such as bacterial product LPS in combination with formyl peptid fMLP leads to an increase in LT formation (LTB_4, trans- and epi-trans isomers, and 5-H(p)ETE) through the activation of 5-LO and $cPLA_2$ (Werz, 2002). Therefore, those cellular assays represent convenient test systems to analyze the potential of inhibitors in physiologic settings. Embelin inhibited the formation of 5-LO products in PMNL stimulated with A23187 or LPS/fMLP concentration-dependently (IC_{50} = 1.7 ± 0.4 (A23187) and 1.3 ± 0.3 μM (LPS/fMLP; Fig. 4.29A). The inhibitory capacity of embelin was similar to the reference inhibitor zileuton (IC_{50} A23187 = 1.1 ± 0.4 μM, IC_{50} LPS/fMLP = 0.9

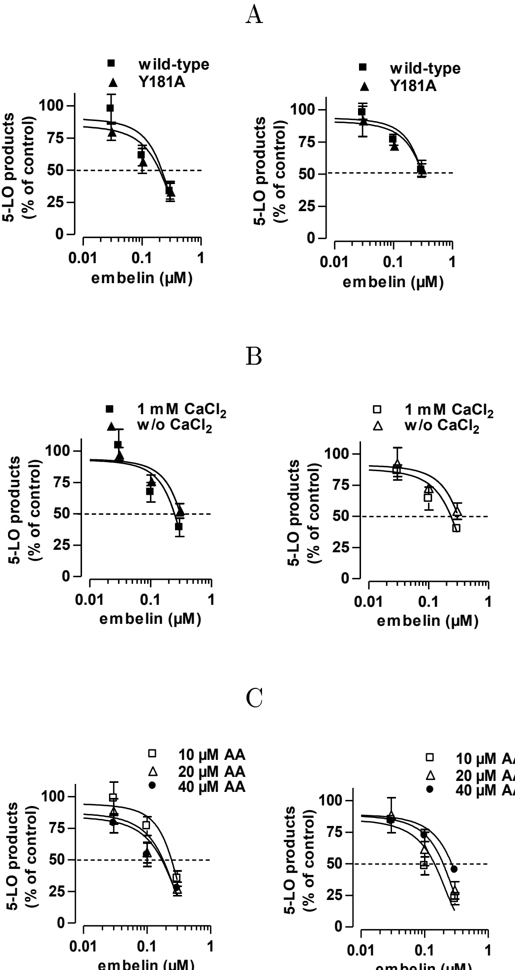

Figure 4.28: Influence of the Y181A mutation of 5-LO on the inhibitory capacity of embelin. (A) 5-LO wild-type and Y181A mutant were incubated with embelin or vehicle (DMSO, 0.1%) at 4 °C for 15 min. Samples were prewarmed for 30 s at 37 °C, 2 mM $CaCl_2$ (left panel) or no $CaCl_2$ (right panel) and 20 μM AA were added and 5-LO product formation was determined after 10 min. (B) Comparison of wild-type (left panel) and Y181A mutant (right panel) 5-LO in presence or absence of $CaCl_2$. (C) Partially purified recombinant 5-LO wild-type (left panel) and Y181A (right panel) stimulated with AA as indicated. Data are expressed as percentage of control (100%), means ± SEM, n = 3-4.

± 0.2 μM; Fig. 4.29B). Stimulation with A23187 or LPS/fMLP might mask the inhibition of $cPLA_2$, which supplies the substrate AA to 5-LO. To exclude this, the substrate AA was supplemented, exogenously. Embelin inhibited 5-LO product synthesis with a slight shift in potency (IC_{50} = 3.5 ± 0.5 μM, Fig. 4.29A). Interestingly, related lipoxygenases such as 15-LO from eosinophils or platelet-type 12-LO from PMNL-adhering platelets were not suppressed following stimulation with A23187 plus AA (Fig. 4.29C). In addition, no influence of embelin was observed on the release of AA from phospholipid vesicles by purified $cPLA_2$ (Table 4.3). Furthermore, the inhibitory capability of embelin was not changed by the addition of the thiol-oxidizing agent diamide together with A23187 plus AA stimulation (not shown). In order to strengthen the results observed in intact cells, the inhibition in human monocytes was also studied. Monocytes were first primed with LPS followed by stimulation with fMLP which leads to formation of LTB_4 and also cysLTs. Embelin inhibited the synthesis of both types of products in monocytes with higher efficien-

cy on the formation of cysLTs (IC_{50} of 0.8 ± 0.04 µM (cysLTs) and 2.0 ± 0.4 µM (LTB_4); Fig. 4.29D). Again as observed for the other assays embelin inhibited LT formation equally potent as the reference inhibitor zileuton (monocytes, LTB_4 IC_{50} = 1.7 ± 0.9 µM; cysLT IC_{50} = 2.7 ± 1.2 µM; Fig. 4.29E).

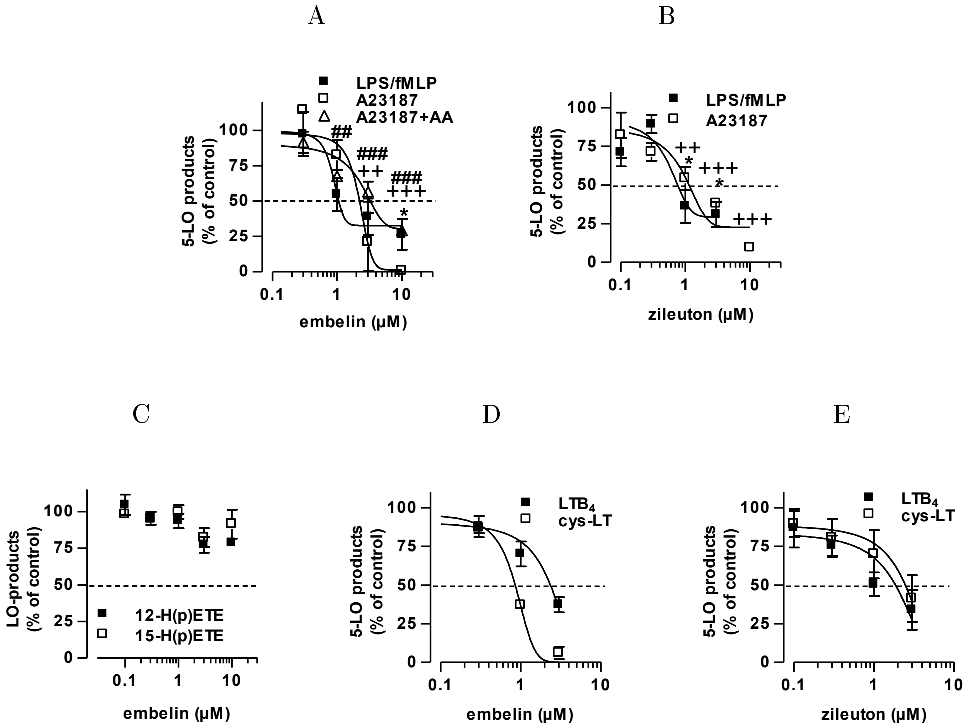

Figure 4.29: Inhibition of 5-lipoxygenase by embelin in cell-based assays. (A,B) PMNL were preincubated with embelin (A), zileuton (B) or vehicle (DMSO, 0.1%) (15 min, 37 °C) and stimulated with 2.5 µM A23187[a] or 2.5 µM A23187 plus 20 µM AA[a] (as indicated) (10 min, 37 °C). Alternatively, PMNL were primed with 1 µg/ml LPS (30 min, 37 °C), 0.3 U/ml Ada (20 min, 37 °C) and stimulated with 1 µM fMLP (5 min, 37 °C). Data are expressed as percentage of control (100%), means ± SEM, n = 3. Embelin (A), fMLP: *, $p < 0.05$ versus 100% control; A23187, ++, $p < 0.01$, +++, $p < 0.001$ versus 100% control; A23187, ##, $p < 0.01$, ###, $p < 0.001$ versus 100% control. Zileuton (B), fMLP, *, $p < 0.05$ versus 100% control of the log-transformed data; A23187, ++,$p < 0.01$, +++, $p < 0.001$ versus 100% control. (C) Effect of embelin on 12-H(p)ETE and 15-H(p)ETE formation in PMNL stimulated with 2.5 µM A23187 plus 20 µM AA[a]. (D,E) Effect of embelin and zileuton on LTB_4 and cysLT formation in monocytes. Human monocytes were primed with 1 µg/ml LPS (5 min, 37 °C), and incubated with embelin (D) and zileuton (E) or vehicle (0.1% DMSO) (15 min, 37 °C) and stimulated with 1 µM fMLP (10 min, 37 °C). Data are expressed as percentage of control (100%), means ± SEM, n = 3. [a]The assay was performed by H. Traber (University of Jena).

It is known that some 5-LO inhibitors (i.e. hyperforin (Feisst et al., 2009)) mediate 5-LO inhibition by the interference with the redistribution of the 5-LO enzyme from the cytosol to the nuclear membrane. Embelin, however, does not inhibit the translocation of 5-LO in A23187-stimulated PMNL (Fig. 4.30A). Besides, modulation of the activation cascades are often observed in relation to the inhibition of LT formation. Therefore, also the influence of embelin on phosphorylation of ERK induced by fMLP was investigated. As shown, embelin did not show any inhibition on the activation of ERK (Fig. 4.30B). Thus, direct interference of embelin with the 5-LO enzyme seems to be most likely.

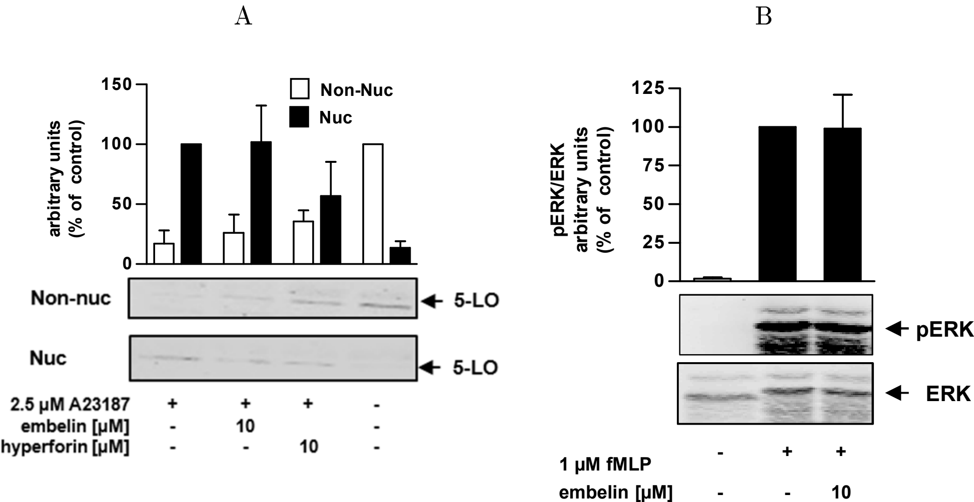

Figure 4.30: Influence of embelin on subcellular localization and ERK phosphorylation. (A) Human PMNL from female donors were preincubated with 10 μM hyperforin, 10 μM embelin or vehicle (0.1% DMSO) for 15 min at 37 °C and stimulated with 2.5 μM A23187 for 5 min at 37 °C. 5-LO distribution was monitored by Western Blot of 5-LO in the nuclear (Nuc) and non-nuclear (Non-nuc) fractions of mild-detergent (0.1% NP-40)-lysed cells. Analysis of protein amounts are given as percentage of controls (the 5-LO band from unstimulated PMNL was set to 100% in Non-nuc, the 5-LO band from A23187-activated PMNL was set 100% in Nuc.; means + SEM. n = 3. (B) Human PMNL from female donors were preincubated with compounds or vehicle control (0.1% DMSO) for 15 min at 37 °C followed by stimulation with 1 μM fMLP for 1.5 min at 37 °C. Data are expressed as percentage of control. Results shown are representatives of 2-3 independent experiments.

4.3.3 Molecular characterization of the inhibition of mPGES-1 by embelin

It is a well-recognized concept that substances which interact with both LT- as well as PGE_2-forming pathways are under certain conditions superior in anti-cancer and anti-infammation treatment than substances acting only on one pathway (Koeberle and Werz, 2009). As many 5-LO inhibitors with lipophilic acid structure also interfere with mPGES-1, interference of embelin with PGE_2 was investigated next. As shown in Fig. 4.31A, embelin strongly inhibited the formation of PGE_2 from PGH_2 in microsomal preparations of IL-1β stimulated A549 cells ($IC_{50} = 0.21 \pm 0.1$ μM). Compared to the control inhibitor MK-886 (Koeberle et al., 2008a) the inhibition was only partially reversible (Fig. 4.31B), indicating that embelin might bind tightly to mPGES-1. Applying substrate concentrations of 1 to 40 μM, PGH_2 revealed an independency from the amount of substrate that is available (Fig. 4.31C). As shown in Fig. 4.31D the potency to inhibit mPGES-1 shifted with a slight loss of potency in the presence of 0.01 or 0.1% of non-ionic detergent triton X-100. Regarding the docking into mPGES-1, the six structural states of embelin (as used for docking into 5-LO (Fig. 4.26) were investigated by the group of Dr. Daniela Schuster from the University of Innsbruck and published in (Schaible et al., 2013b). Embelin was predicted to interfere with the site adjacent to the binding site of the co-substrate GSH with several basic residues (e.g., Arg73A, Arg70C, and Arg73C), hydrophobic residues (e.g., Leu69A, Met76A, Leu69B, Met76B, and Met76C) as well as with Tyr117C and GSH, which participate in hydrogen bonds (Fig. 4.31E). Basically, the other redox states and deprotonated embelin species showed a comparable orienta-

tion in proximity to GSH, as embelin.

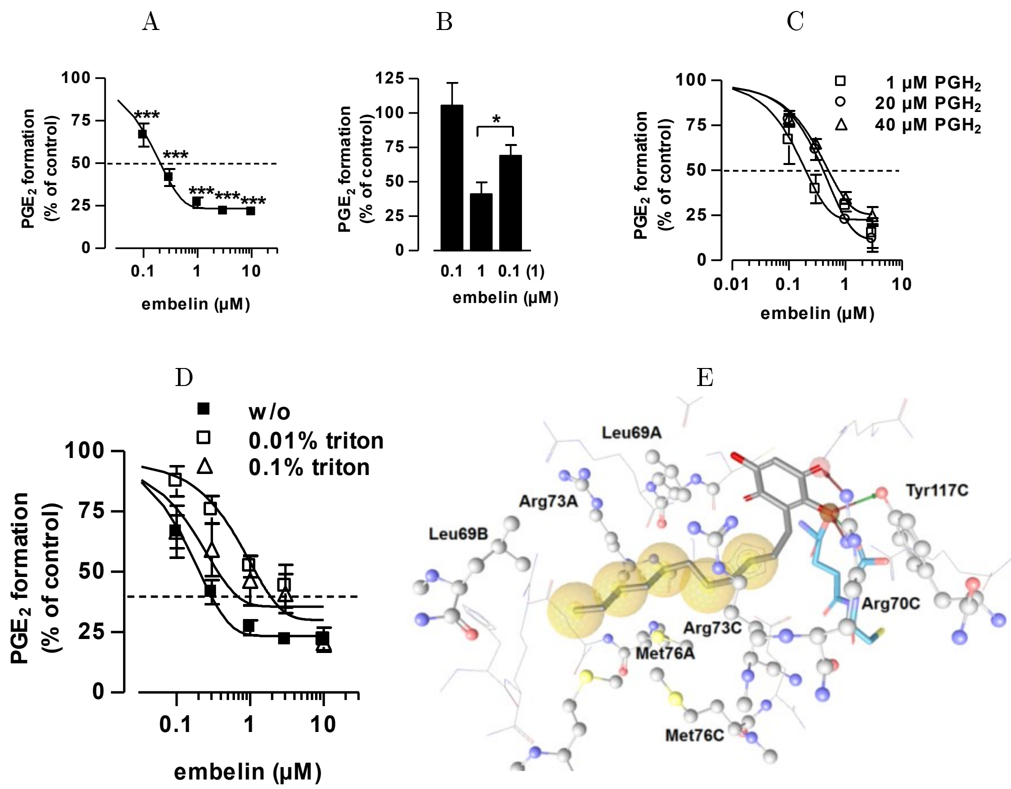

Figure 4.31: Inhibition of mPGES-1 by embelin. (A) Microsomal preparations of IL-1β stimulated A549 cells were preincubated with embelin or vehicle (0.5% DMSO) (15 min, 4 °C) and the reaction was started by addition of 20 μM PGH_2 (1 min, 4 °C). MK-886 (10 μM) was used as a reference inhibitor that caused 77.2 ± 1.1% inhibition. ***, $p < 0.001$ versus 100% control.[a] (B) Inhibition of mPGES-1 by embelin is partially reversible. Microsomal preparations of IL-1β stimulated A549 cells were preincubated with 0.1 or 1 μM embelin (15 min, 4 °C). An aliquot of the sample containing 1 μM embelin was diluted 10-fold ("0.1 (1)") and the other aliquot left untreated. 20 μM PGH_2 was added to start the reaction and stopped after 1 min at 4 °C as described above. *, $p < 0.05$ versus inhibition without dilution. (C) Inhibition of mPGES-1 by embelin was determined at different PGH_2 concentrations (1, 20 and 40 μM PGH_2). (D) Microsomal preparations of IL-1β stimulated A549 cells were incubated with embelin in the presence or absence of 0.01 or 0.1% Triton X-100, 20 μM PGH_2 was added and mPGES-1 activity was assesed. Data are expressed as percentage of control (100%) and given a means ± SEM, n = 3-4. (E) Molecular docking simulations of embelin (grey) into mPGES-1 were performed by the group of Dr. Daniela Schuster, University of Innsbruck, Austria and published in (Schaible et al., 2013b). While embelin is oriented within the binding site by hydrogen bonds with Arg-70, Tyr-117, and GSH (blue) the n-undecanyl chain forms hydrophobic interactions with lipophilic amino acids. Chemical interactions between embelin and the mPGES-1 protein are color-coded: yellow sphere – hydrophobic interaction, red arrow – hydrogen bond acceptor, green arrow – hydrogen bond donor. [a]The assay was performed by K. Fischer (University of Jena).

4.3.4 Evaluation of further targets of embelin related to eicosanoid pathway

Next, other relevant targets within eicosanoid biosynthesis such as $cPLA_2$, COX-1 and COX-2 were analyzed. As shown in Table 6, embelin did not inhibit COX-1 (cell-free or in platelets) as well as isolated COX-2 or $cPLA_2$ at 10 μM.

Table 4.3: Effects of embelin on the activity of COX-1/2 and $cPLA_2$. Embelin (10 μM) or reference inhibitors (at the indicated concentrations) were added to the respective enzymes or freshly isolated human platelets 15 min prior induction of the enzyme reaction. Data (means ± S.E., n = 3) are expressed as percentage of the remaining activity of the uninhibited vehicle (0.1% DMSO) control (100%). indo = indometacin. Some assays were performed by [a]B. Schmalwasser and [b]M. Melzer (University of Jena).

enzyme/ assay	embelin (10 μM) % remaining activity	reference control % remaining activity
COX-1, cell-free	105.7 ± 7.1%	23.9 ± 2.6% (indo, 10 μM)
COX-1, platelets	89.5 ± 7.3%	22.4 ± 9.6% (indo, 10 μM)
COX-2, cell-free[a]	102.2 ± 6.5%	43.0 ± 2.7% (indo, 10 μM)
$cPLA_2$, cell-free[b]	98.6 ± 10.2%	23.8 ± 5.0% (RSC-3388, 5 μM)

4.3.5 Antioxidant and radical scavenging activity and cytotoxicity of embelin

Embelin exhibits partial antioxidant capacities (Joshi et al., 2007; Mahendran et al., 2011a). This is a feature many 5-LO inhibitors have in common (Pergola and Werz, 2010). In an assay using DPPH, a stable radical, embelin shows partial scavenging of DPPH (Fig. 4.32A). In contrast to the reference compounds ascorbic acid and L-cysteine embelin was not able to scavenge DPPH completely, which has also been reported before (Joshi et al., 2007; Mahendran et al., 2011a). To evaluate the antioxidant potential of embelin in a cellular assay human PMNL were loaded with a peroxide-sensitive DCF-DA and after preincubation with embelin the formation of ROS was induced with PMA. As shown in Fig. 4.32B embelin was able to block ROS formation at a concentration of 10 μM to the basal level whereas lower concentrations were without effect. Finally, cytotoxic effects of embelin were assayed reproducing the incubation periods of the cellular assays. Incubation of PMNL or monocytes for 30 min at 37 °C with 10 μM embelin did not significantly impair cell viability as assessed by trypan blue exclusion (PMNL: 88.4 ± 0.8%, monocytes: 83.1 ± 0.4% viable cells treated with embelin compared to 91.1 ± 1.0% (PMNL) and 88.9 ± 0.2% (monocytes) viable control cells incubated with 0.1% DMSO).

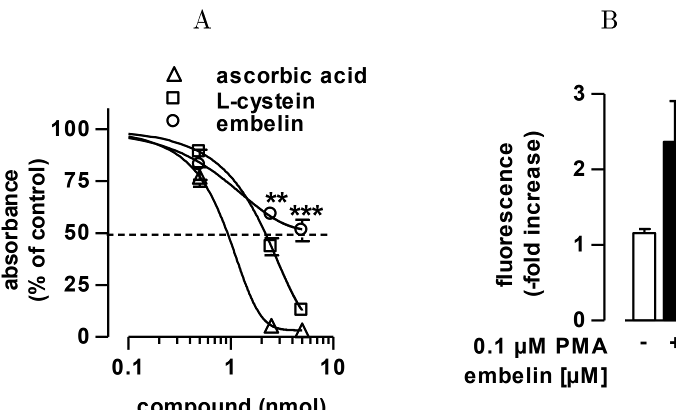

Figure 4.32: Radical scavenging properties and influence of embelin on ROS formation in PMNL. (A) Radical scavenging properties. Embelin was incubated with 5 nmol DPPH in 100 μl of ethanol for 30 min at RT and the absorbance was measured at 520 nm. Ascorbic acid and L-cysteine were used as controls. Values are given as percentage of control (100%) mean $\pm$ S.E., n = 3. Embelin, **, $p < 0.01$, ***, $p < 0.001$ versus 100% control. (B) Influence of embelin on ROS formation. PMNL were pre-incubated with embelin for 15 min, loaded with the fluorescent dye DCF-DA and stimulated with 0.1 μM PMA. The relative increase in fluorescence was determined after at 37 °C after 360 s. Data (means + SEM, n = 3) are expressed as –fold increase (t = 0 and t = 360 s). This assay was performed by H. Traber (University of Jena).

Taken together, embelin is a so-called dual inhibitor of 5-LO and mPGES-1 with potent and direct interference with the respective enzymes resulting in reduced product formation. However, embelin does not inhibit related eicosanoid forming enzymes such as COX-1/2, 12-LO and 15-LO. Furthermore, embelin does not interfere with 5-LO activating signal cascades (translocation, MAPK pathway) nor inhibit isolated $cPLA_2$. Embelin exhibits partial antioxidant capacities which however, do not correlate with the mode of inhibition at 5-LO. Based on the findings with this natural derived compound new derivatives were designed in collaboration with Dr. Rosanna Filosa, University of Salerno, Italy to study SARs and to increase the inhibitory potential in the cells and in blood assays.

4.4 Investigation of RF-Id as 5-LO inhibitor

RF-Id (3-((decahydronaphthalen-6-yl)methyl)-2,5-dihydroxycyclohexa-2,5-diene-1,4-dione) (Fig. 4.33) was identified as lead compound among a series of novel 1,4-benzoquinone chemotypes that were generated to intervene with LT biosynthesis (Filosa et al., 2013).

Figure 4.33: Chemical structure of RF-Id

Interestingly, RF-Id inhibited 5-LO product formation in A23187 stimulated PMNL with a higher potency ($IC_{50} = 0.58$ μM) compared to the isolated enzyme (IC_{50} = 11

μM) (Filosa et al., 2013). Therefore, it was of interest to elucidate the mechanisms behind the increased potency in cellular assays. This study was published in (Schaible et al., 2014). First, the efficiency of RF-Id to interfere with LT formation was evaluated applying different assays. As shown in Fig. 4.34A, addition of exogenous AA (20 or 40 μM) did not change the inhibitory capacity of RF-Id. Furthermore, RF-Id potently inhibited LTB_4 and cys-LT formation under pathophysiological relevant conditions (LPS/fMLP) in neutrophils and monocytes (Fig. 4.34B, C). As already reported for isolated 5-LO enzyme, inhibition of RF-Id was lost in homogenates of neutrophils compared to intact cells (Fig. 4.34D).

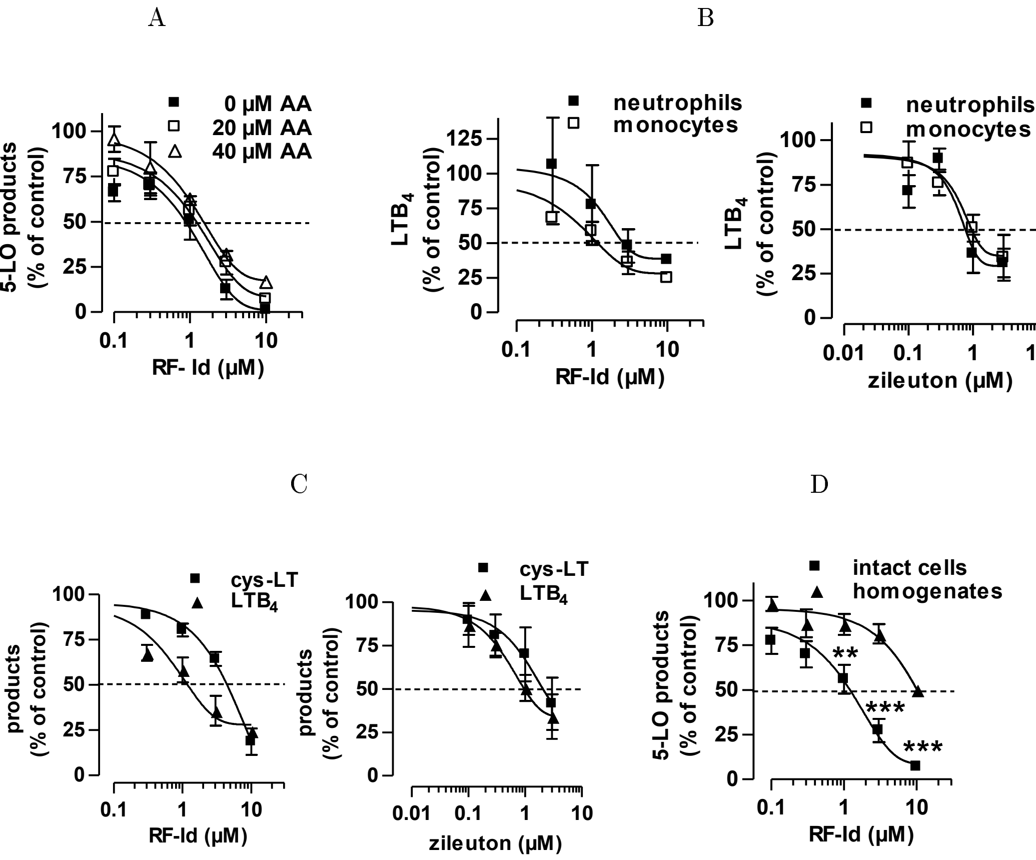

Figure 4.34: Inhibition of LT formation by RF-Id in cellular assays. (A) Neutrophils were preincubated with RF-Id (15 min, 37 °C) and stimulated with 2.5 μM A23187 plus AA as indicated (10 min, 37 °C). Data are means ± SE; n = 3.[a] (B) Neutrophils were primed with 1 μg/mL LPS (15 min, 37 °C), after 5 min 0.3 U/mL Ada was added, and cells were then preincubated with the compounds (RF-Id, left panel; zileuton, right panel) for 15 min at 37 °C prior to stimulation with 1 μM fMLP (5 min, 37 °C). Monocytes were primed with 1 μg/mL LPS (5 min, 37 °C), then compounds were added for 15 min, and cells were stimulated (10 min, 37 °C) with 1 μM fMLP. Data are means ± SE; n = 3. (C) Effects of RF-Id on LTB_4 and cys-LT formation in monocytes. Cells were stimulated as described under B. LTB_4 was quantified by HPLC and cys-LT levels by EIA in supernatants, respectively. Data are means ± SE; n = 3. (D) Intact neutrophils were preincubated with RF-Id (15 min, 37 °C) and stimulated with 2.5 μM A23187 plus 20 μM AA (10 min, 37 °C). Neutrophil homogenates were incubated with RF-Id (15 min, 4 °C). After addition of 1 mM ATP, samples were warmed up (30 s, 37 °C) and stimulated with 2 mM $CaCl_2$ and 20 μM AA (10 min, 37 °C). **, $p < 0.01$, ***, $p < 0.001$ vs vehicle control (0.1% DMSO). Data are expressed as percentage of control (0.1% DMSO), means ± SE; n = 3. [a]The assay was performed by H. Traber (University of Jena).

The higher efficiency in cells was not due to cytotoxic effects as shown by a trypan blue exclusion assay for neutrophils and by a MTT assay for monocytes after 1 and 24 h of incubation, respectively (Fig. 4.35A). RF-Id did not inhibit the subcellular translocation of 5-LO (Fig. 4.35B).

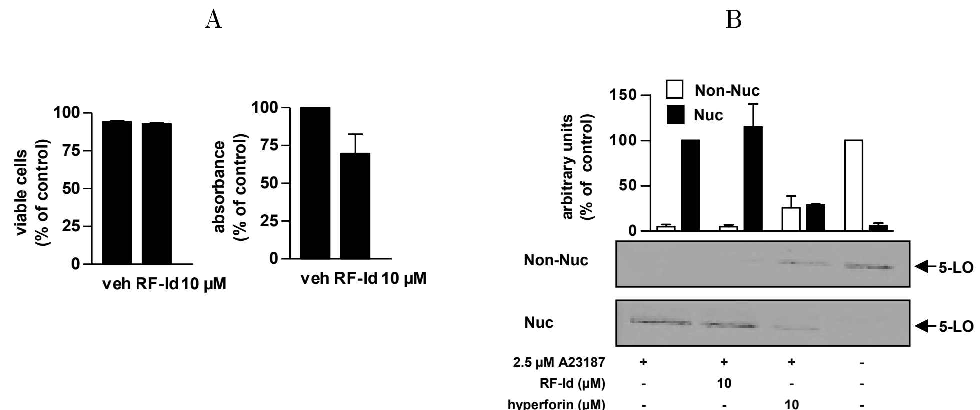

Figure 4.35: Effects of RF-Id on cell viability and subcellular localization of 5-LO. (A) Neutrophils were incubated with RF-Id (10 μM) or vehicle (veh, 0.1% DMSO) for 30 min at 37 °C and trypan blue exclusion was analyzed using a Vi-cell counter (left panel). Monocytes were incubated for 24 h with RF-Id or vehicle (veh, 0.3% DMSO)and the viability of the cells was analyzed by MTT assay (right panel). Data are expressed as percentage of vehicle, means + SEM, n = 3. (B) Effects of RF-Id and hyperforin on 5-LO subcellular localization in neutrophils following stimulation with 2.5 μM A23187 for 5 min at 37 °C. The distribution of 5-LO was analyzed by Western blot in the nuclear and non-nuclear fraction of mild detergent (0.1% NP-40)-lysed cells. One representative experiment of three independent experiments is shown. Data are expressed as percentage of control (the 5-LO band from unstimulated PMNL was set to 100% in Non-nuc, the 5-LO band from A23187-activated PMNL was set 100% in Nuc.; means + SEM. n = 3.

In contrast to embelin (4.3.1), RF-Id was able to inhibit LT synthesis in homogenates of neutrophils after restoring the reducing milieu by addition of DTT (1 mM) with a shift of the IC_{50} value from 10 to 1.7 μM (Fig. 4.36A). Thus, the inhibitory potential was similar as in the cellular assays. This suggests that RF-Id requires the reducing milieu of the cell in which the abundance of thiols (like GSH) is responsible for the reduction of 1,4-benzoquinones to the hydroquinone forms (Ohkawa et al., 1991a). In order to investigate this in a cellular assay, the thiol-oxidizing agent diamide was added to neutrophils, which leads to an elevation of the cellular oxidative tone. As shown, the 5-LO inhibitory capacity of RF-Id was counteracted by the addition of diamide whereas this was not found for zileuton. The IC_{50} shifted from 1.5 to 10 μM, which was also found in non-cellular assays (Fig. 4.36B). Additionally, the ability of RF-Id to scavenge the stable radical DPPH was investigated. RF-Id did not scavenge DPPH (Fig. 4.36C). Interestingly, the ability of RF-Id to inhibit purified 5-LO could also be increased by the addition of 1 mM DTT to the purified preparations (Fig. 4.36A).
Taken together, in comparison to embelin the redox status is crucial to for 5-LO inhibition by RF-Id. Though both compounds share structural similarities, the mode of inhibition on the mechanistic level is different. Reduction of RF-Id by the addition of DTT to the hydroquinone form leads to an increase in inhibitory potential in cell-free assays.

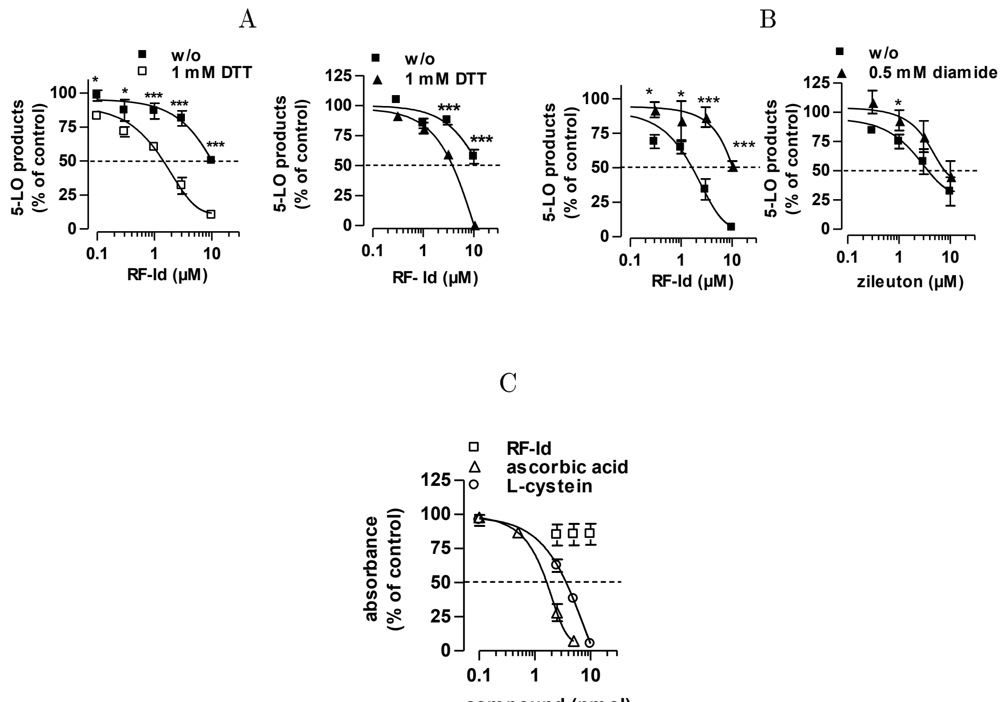

Figure 4.36: Influence of the redox state on 5-LO inhibition by RF-Id. (A) PMNL homogenates (left panel) or purified 5-LO (right panel) were preincubated with RF-Id or vehicle (0.1% DMSO) (15 min, 4 °C). 5 min prior to stimulation, 1 mM DTT was added as indicated. Samples were warmed up for 30 s at 37 °C and stimulated with 2 mM $CaCl_2$ and 20 μM AA (10 min, 37 °C). *, $p < 0.05$, ***, $p < 0.001$ DTT treatment vs w/o. Data are expressed as percentage of vehicle (0.1% DMSO) control, means ± SEM. n = 3. (B) To intact neutrophils, 500 μM diamide or vehicle (0.1% DMSO) was added 7.5 min before addition of RF-Id (left panel) or zileuton (right panel) at 37 °C. After addition of compounds for another 7.5 min at 37 °C, cells were stimulated with 2.5 μM A23187 plus 20 μM AA for 10 min at 37 °C. *, $p < 0.05$, ***, $p < 0.001$ diamide treatment vs w/o. Data are expressed as percentage of control, means ± SEM, n = 3. (C) Reduction of the DPPH radical (5 nmol) by, RF-Id, ascorbic acid and L-cystein. Data are means + SE; n = 3.

In collaboration with Dr. Daniela Schuster (University of Innsbruck, Austria) a docking simulation for RF-Id was performed in order to investigate the molecular mechanism responsible for 5-LO inhibition by RF-Id and to study the effects of the reduced hydroquinone form (Fig. 4.37) ((Schaible et al., 2014)). Furthermore, the enantiomers of each redox-form were docked into the crystal structure of 5-LO (PDB entry 3o8y)(Gilbert et al., 2011). All ten docking poses of each structure were very similar, suggesting a reliable pose prediction (Jones and Willett, 1995). The hydrophobic rings fill the hydrophobic channel running by the catalytic iron, where the oxidation of AA would take place in the non-inhibited state. The hydroquinone ring is coordinated between several amino acids and water molecules mediating additional hydrogen bonds with the protein. While no difference between the enantiomers of RF-Id could be observed, the redox state of the compound had a considerable effect on the predicted interaction patterns. For the quinone state several interactions were calculated, depending on the exact orientation of the ring: Hydrogen bonds with Gln-557, His-367, Tyr-181, and Asn-425 were observed, as well as interactions with two the water molecules H2O855 and H2O724. In the hydroquinone state even more interactions are observed, because the additional hydroxyl-groups can act both

as donors and acceptors of hydrogen bonds. Interactions are formed with Gln-557, Tyr-181, Gln-363, Asn-425, His-367, and the two water molecules.

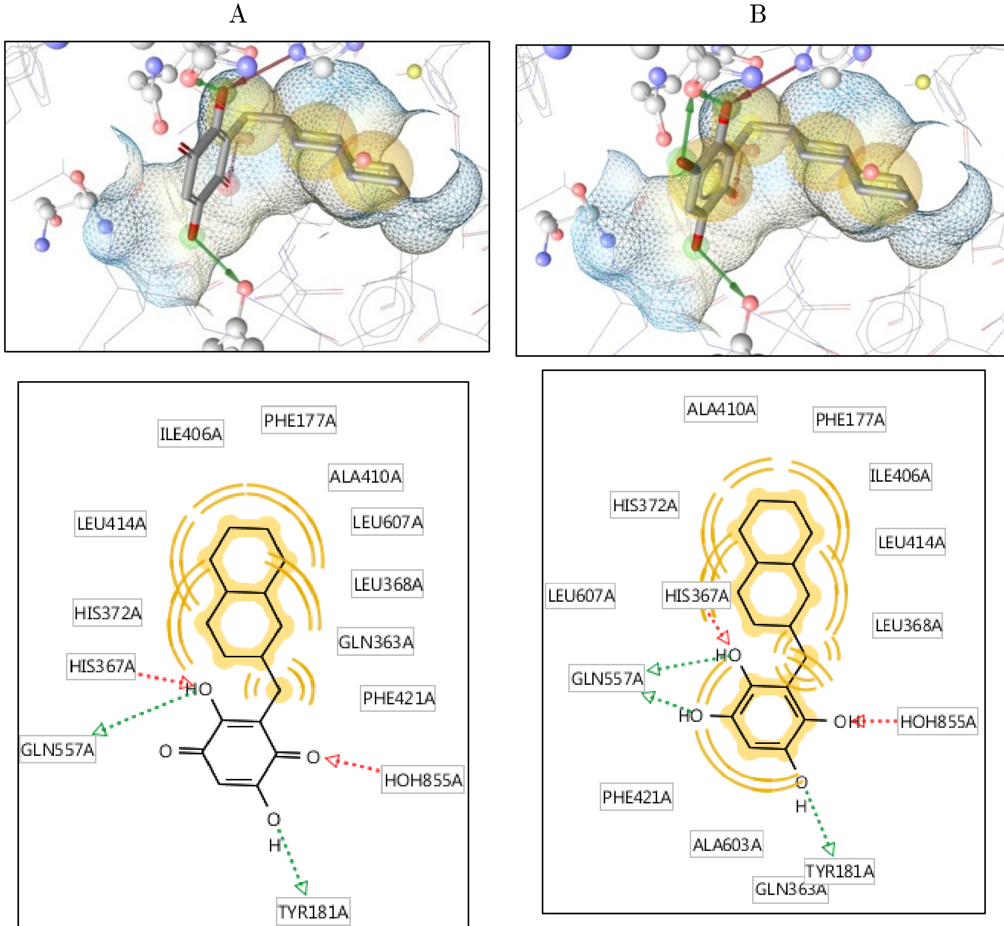

Figure 4.37: Docking poses for RF-Id in the oxidized and reduced state. (A) Docking pose of RF-Id in the oxidized state within the binding pocket. Interactions with Gln-557, Tyr-181, His-367 and H2O855 are shown. The hydrophobic part of the molecules fills the substrate channel. (B) shows RF-Id in the reduced state within the binding pocket. Interactions with Gln-557, Tyr-181, His-367 and H2O855 are shown. Data were generated by the group of Dr. Daniela Schuster, University of Innsbruck, Austria and published in (Schaible et al., 2014).

RF-Id did not interfere with other 5-LO related targets such as, COX-1 or $cPLA_2$ (Table 4.4). 12- and 15-H(p)ETE were not inhibited in neutrophil homogenates with or without addition of DTT (Fig. 4.38). However, RF-Id modestly inhibited COX-2 in IL-1β treated A549 cells with an IC_{50} of 7.3 ± 0.5 μM. On the other hand, product formation (12-HHT) from isolated COX-2 was not reduced by RF-Id (Table 4.4).

Table 4.4: Effects of RF-Id on COX-1/2, cPLA$_2$ and mPGES-1. RF-Id (10 μM) or reference inhibitors (at the indicated concentrations) were added to the respective enzymes or freshly isolated human platelets 15 min prior induction of the reaction. Data (means ± S.E., n = 3) are expressed as percentage of the remaining activity of the uninhibited vehicle (0.1% DMSO) control (100%). indo = indometacin, cele = celecoxib. Some assays were performed by [a]B. Schmalwasser and [b]K. Fischer.

enzyme/ assay	RF-Id (10 μM) % remaining activity or IC_{50}	reference control % remaining activity
COX-1, cell-free[a]	89.6 ± 13.6%	22.2 ± 2.6% (indo, 10 μM)
COX-1, platelets	93.8 ± 3.3%	5.9 ± 3.1% (indo, 10 μM)
COX-2, cell-free[a]	76.4 ± 7.6%	43.0 ± 2.7% (indo, 5 μM)
COX-2, A549 cells	7.3 ± 0.5 μM	16.9 ± 0.5%, (indo 10 μM)
		25.2 ± 4.9% (cele 5 μM)
cPLA$_2$, cell-free	79.9 ± 8.2%	11.6 ± 5.9% (RSC-3388, 5 μM)
mPGES-1, cell-free[b]	71.6 ± 11.9%	26.7 ± 8.0% (MK-886, 10 μM)

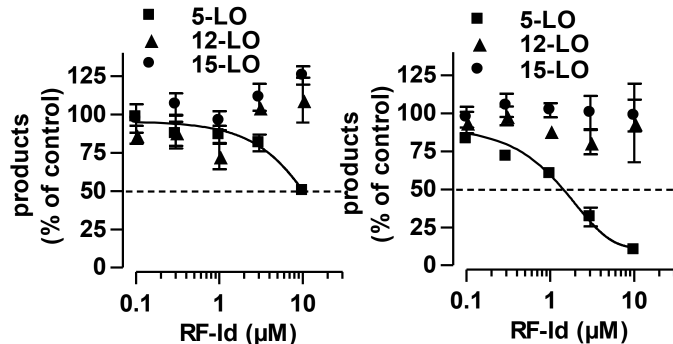

Figure 4.38: Effect of RF-Id on 12-LO and 15-LO in neutrophil homogenates. Neutrophil homogenates (corresponding to 5 × 10^6 cells) were preincubated with RF-Id or vehicle (0.1% DMSO) for 15 min at 4 °C (left panel). After 7.5 min 1 mM DTT was added for 7.5 min (right panel). Samples were prewarmed for 30 s at 37 °C and stimulated with 2 mM $CaCl_2$ and 20 μM AA for 10 min at 37 °C. Data are percentage of vehicle control, means ± SEM. n = 3.

Encouraged by the inhibitory capacity of RF-Id in cellular experiments, RF-Id was analyzed for inhibition of 5-LO in human blood and tested in *in vivo* models of inflammation in mice. In heparinized blood stimulated with LPS and fMLP, LT formation was inhibited by RF-Id with an IC_{50} of 4.1 ± 0.6 μM (Fig. 4.39A). The *in vivo* experiments were conducted in the lab of Prof. Bruno D'Agostino, University of Naples, Italy (published in (Schaible et al., 2014). For the carrageenan-induced paw edema in mice, intraplantar injection of carrageenan led to a massive swelling of the paw after 2 h. This inflammatory reaction could successfully be reduced by RF-Id which was injected *i.p.*, 30 min prior to carrageenan injection. Already at a dose of 1 mg/kg the effects were maximal and evident at all time points. In the air pouch experiment, LTB_4 as chemotactic agent led to the migration of immune cells. RF-Id significantly impaired cell migration at a concentration of 0.1 mg/kg after injection of zymosam.

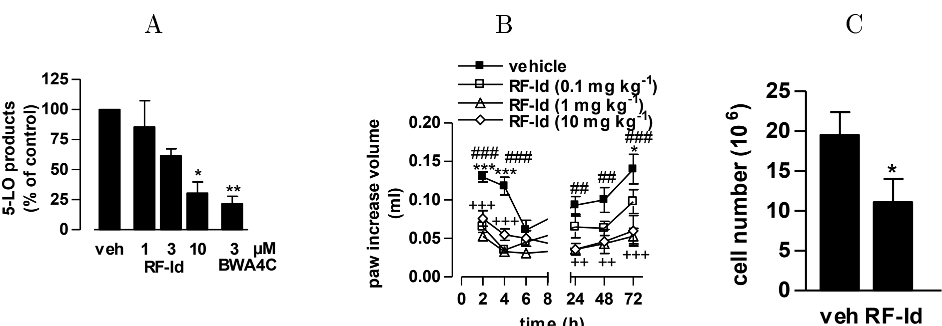

Figure 4.39: Effect of RF-Id on LT formation in human blood. (A) Human whole blood was primed with 1 μg/mL LPS (15 min, 37 °C), treated with the test compounds or veh (0.1% DMSO as vehicle) (15 min, 37 °C), and then stimulated with 1 μM fMLP (15 min, 37 °C) (right panel). *,$p < 0.05$, **,$p < 0.01$ vs vehicle control. Data are expressed as percentage, means + SE; n = 3. (B) Carrageenan-induced edema. Mice were divided in 3 groups (n=6) and received *i.p.* administration of RF-Id (0.1 – 1 - 10 mg/kg) 30 min before subplantar injection of 50 μl of carrageenan (1%, w/v). Paw volume was measured using a hydroplethismometer, specially modified for small volumes, immediately before the subplantar injection (basal value) and 2, 4, 6, 24, 48 and 72 h thereafter. Data are means ± SEM, n=6. 0.1 mg kg-1: *, $p < 0.05$; ***, $p < 0.001$; 1 mg/kg: ##, $p < 0.01$; ###, $p < 0.001$; ++, $p < 0.01$; +++, $p < 0.001$. (C) Cell migration in air pouches of mice. Mice were divided in 2 groups (n=6) and received *i.p.* administration of RF-Id (0.1 mg/kg) or veh (vehicle, DMSO). After 30 min, air pouches were developed by subcutaneous injection of sterile air into the back of mice. After 4 h of zymosan (1% w/v) injection, mice were sacrificed by CO_2 exposure and exudate in the pouch was collected and the total leukocyte count was evaluated by optical microscopy in the cell suspension diluted with Turk's solution. Data are means + SEM, n=6. *, $p < 0.05$. Experiments shown in (B) and (C) were done by the group of Prof. Bruno D'Agostino, University of Naples and published in (Schaible et al., 2014).

4.4.1 Comparison of the interference with leukotriene formation by embelin versus RF-Id

Considering the similarity of structures of embelin and RF-Id, the effects of both on LT formation are summarized (Table 4.5). Main differences are the high activity of embelin at the isolated 5-LO enzyme (embelin, IC_{50} = 0.06 μM versus RF-Id, IC_{50}= 11 μM) and mPGES-1 (embelin, IC_{50}= 0.21 μM versus RF-Id no inhibition at 10 μM). While embelin lost activity in cellular assays, RF-Id was more potent. Interference with the redox system of the cell (addition of diamide in cellular assays or DTT in cell-free assays) was only evident for RF-Id and not for embelin.

Table 4.5: Summary of the effects of embelin and RF-Id in the different assays.

enzyme/ assay	emblin IC_{50} (μM)	RF-Id IC_{50} (μM)
5-LO, cell free (20 μM AA)	0.06	11
5-LO, PMNL homogenates (20 μM AA)	0.11	10
effect of DTT in homogenates	×	✓
effect of redox-form of the compound on docking simulation	×	✓
intact PMNL (2.5 μM A23187)	1.7	0.9
intact PMNL (2.5 μM A23187/20 μM AA)	3.5	0.9
intact PMNL (LPS/fMLP)	1.3	2.6
intact monocytes (LPS/fMLP; LTB_4)	0.8	1.7
intact monocytes (LPS/fMLP; cysLT)	2.0	4.2
effect of diamide in intact PMNL	×	✓
12-H(p)ETE formation PMNL	×	×
15-H(p)ETE formation PMN L	×	×
effect on 5-LO translocation	×	×
whole blood (LPS/fMLP)	×	4.1
radical scavenging	partial	×
ROS formation in PMNL	✓	n.d.
mPGES-1	0.21	×
COX-1, platelets	×	×
COX-2, A549	induction	7.3
COX-1, cell-free	×	×
COX-2, cell-free	×	×
$cPLA_2$, cell-free	×	×

× = no effect (10 μM of substance); ✓ = effect;
n.d. = not determined

4.5 Inhibition of 5-LO by hydroxybenzoquinones

In a previous study aiming to assess the biological activity of synthetic 1,4-benzoquinones, a series of 2,5-dihydroxylated 1,4-benzoquinones with lipophilic and bulky alkyl- or aryl-substituents in 3-position, were identified as potent inhibitors of 5-LO (Filosa et al., 2013). The high potency of embelin on 5-LO and the promising *in vivo* efficiency of RF-Id led to the idea to further modify the structures and thus to improve the inhibitory potential as well as to investigate SARs. The compounds were synthesized by Dr. Rosanna Filosa (University of Salerno, Italy).

4.5.1 SARs of hydroxybenzoquinones

Though the synthesized structures and variations seem to be quite obvious and simple, the read-outs from the different assay systems were surprising. Starting from embelin, the alkyl chain length in position 3 (*n*-undecyl residue) was varied by introducing saturated linear C4, C6, C10, C12 and C16-alkyl residues or isoprenoid side chains with 2 or 3 prenyl moieties, respectively (Table 4.6). All derivatives except compound 1 which carries an *n*-butyl residue inhibit the formation of 5-LO products

in cell-free enzyme assay with IC_{50} values ranging from 0.17 to 4 μM. However, none of the derivatives was superior to embelin (IC_{50} = 0.06 μM). Interestingly, a relationship between the inhibitory potential and the length of the alkyl chain can be found with an optimum for the *n*-undecyl residue of embelin. Compounds with C10, C12 and C16 chains (compound 3, 5 and 6) inhibited isolated 5-LO equally well in the submicromolar range with IC_{50} values between 0.17 and 0.19 μM. Shortening the chain to 6 carbon atoms (compound 2, C6) led to a tremendous loss of potency (about 22-fold). Insertion of prenyl residues gave compounds 7 and 8 and led to a loss of inhibitory efficacy compared to embelin (IC_{50} = 1.8 and 2.5 μM, respectively). Next, the capabilty of the compounds to interfere with 5-LO product formation in PMNL stimulated with Ca^{2+}-mobilizing agent A23187 or with A23187 plus supplementation of AA was investigated. All compounds showed lower potency in inhibiting the synthesis of 5-LO products compared to isolated enzyme preparations. Besides embelin only compound 3 and 5 showed IC_{50} values < 10 μM in cellular assays. Again none of the compounds was superior to embelin.

Next, one or two hydroxyl groups were methylated in position 2 and 5, respec-

Table 4.6: Effects of 2,5-hydroxy-1,4-benzoquinones on inhibition of 5-LO. *Embelin.

No.	(structure: 2,5-dihydroxy-1,4-benzoquinone with R; O, OH, HO, R)	5-LO activity IC_{50} [μM] (remaining activity at 10 μM) cell-based A23187	A23187+AA	cell-free AA
1	R=	>10 μM (93.5± 4.4%)	>10 μM (92.8± 4.2%)	>10 μM (65.4± 14.2%)
2	R=	>10 μM (133.5± 19%)	>10 μM (79.9± 6.6%)	4.0± 1.1
3	R=	2.0± 0.3	7.1± 1.2	0.18± 0.01
4*	R=	1.7± 0.4	3.5± 0.5	0.06± 0.01
5	R=	4.6± 1.3	3.9± 1	0.17± 0.03
6	R=	>10 μM (88.0± 16.3%)	>10 μM (80.5± 5.7%)	0.19± 0.04
7	R=	>10 μM (114.3± 4.5%)	>10 μM (81.9± 10.8%)	1.8± 0.2
8	R=	>10 μM (85.8± 3.9%)	>10 μM (66.6± 2.8%)	2.5± 1.4

tively (Table 4.7 and Table 4.8). Interestingly, the capability of the *O*-mono- and *O,O'*-dimethylated analogues to inhibit 5-LO product synthesis in PMNL stimulated with A23187 (plus AA) was improved versus non-methylated analogues. The potency against 5-LO activity in the cell-free test system was reduced compared to the derivatives carrying free hydroxyl groups with higher impact by monomethylation. For example, compound 10, 14 and 15 did not inhibit isolated 5-LO by

more than 50% at 10 μM. For the *O,O'*-dimethylated analogues higher potency against the isolated enzyme was observed as for the monomethylated derivatives (2- to 3-fold). The highest efficiency was again found for derivatives with chain lengths between C10 and C16 (IC_{50} = 0.6 – 1.8 μM). In neutrophils, *O*-monomethylated compounds with linear C10, C11- and C12-alkyl residues (11, 12 and 13) were again the most active and reached IC_{50} values between 0.5 and 0.7 μM (A23187) and 1.1 to 2.7 μM (A23187 plus AA). In parallel to the results observed for isolated 5-LO, *O,O'*-dimethylated C10-, C11- and C12- analogues 19, 20, and 21 showed even lower values with IC_{50} = 0.2 to 0.6 μM for A23187 stimulation and 0.5 to 1.7 μM for stimulation with A23187 plus AA. Compound 22 carrying a chain with a length of 16 carbon atoms showed loss of activity in cellular assays (IC_{50} = 3.5 μM, A23187 plus AA versus 1.6 μM) at isolated 5-LO). After stimulation with A23187 only no inhibition was found at 10 μM which is not readily understood. Also for the prenylated derivatives the cellular potential improved with *O*-monomethylation (15 and 16) and even more with *O,O'*-dimethylation (23 and 24) versus the unmethylated derivatives (7 and 8). In summary, the inhibitory potential of the compounds in PMNL increases with the degree of methylation (dimethylation or monomethylation) and the length of the chain in position 3 at the 2,5-dihydroxy-1,4-benzoquinone backbone (with highest efficiency between C10 and C16).

In a next step, the 2,5-dimethoxy-1,4-benzoquinone core was replaced by a 4,5-

Table 4.7: Effects of 2-hydroxy-5-methoxy-1,4-benzoquinones on inhibition of 5-LO.

No.	5-LO activity IC_{50} [μM] (remaining activity at 10 μM)		
	cell-based		cell-free
	A23187	A23187+AA	AA
9 R=	>10 μM (86.0± 6.0%)	>10 μM (95.7± 7.3%)	>10 μM (70.5±17.1%)
10 R=	>10 μM (68.9± 16.3%)	>10 μM (64.1± 8.0%)	>10 μM (71.1± 5.3%)
11 R=	0.59± 0.1	2.7± 0.7	4.3± 0.3
12 R=	0.68± 0.1	1.3± 0.4	3.8± 0.5
13 R=	0.56± 0.04	1.1± 0.2	0.74± 0.1
14 R=	1.0± 0.6	1.1± 0.8	> 10 μM (60.1± 8.7%))
15 R=	4.1± 1.1	>10 μM (65.9± 6.0%)	>10 μM (73.6± 8.0%)
16 R=	1.9± 0.05	2.4± 0.8	5.6± 0.04

methoxy-1,2-benzoquinone backbone (*ortho*-quinone structures) leading to compounds 25 – 32 (Table 4.9) decorated with the same linear and prenylated chains

Table 4.8: Effects of 2,5-methoxy-1,4-benzoquinones on inhibition of 5-LO.

No.	5-LO activity IC_{50} [μM] (remaining activity at 10 μM)		
	cell-based		cell-free
	A23187	A23187+AA	AA
17 R=	3.4± 1.4	7.9± 1.0	3.3± 0.9
18 R=	0.32± 0.2	1.4± 0.4	2.6± 1.2
19 R=	0.60± 0.4	1.7± 0.9	1.8± 0.7
20 R=	0.20± 0.02	0.59± 0.1	0.93± 0.1
21 R=	0.63± 0.4	0.49± 0.1	0.61± 0.08
22 R=	> 10 μM (80.1± 9.1%)	3.5± 0.9	1.6± 0.2
23 R=	0.45± 0.2	1.6± 0.2 (65.9± 6.0%)	3.3± 0.7 (73.6± 8.0%)
24 R=	0.31± 0.1	1.2± 0.2	1.7± 0.7

as for the other groups. The shift of the residues led to increased inhibitory activity compared with the *para*-analogues. Again, derivatives with C10 to C12 chains were the most potent with high potency in the cellular assay (IC_{50} values: 27: 0.04 μM; 28: 0.06 μM; 29: 0.03 μM). For the compound with the highest activity (29), a shift of 21-fold was observed compared to its related compound 21. In addition, the potency against the isolated 5-LO enzyme was increased compared to the 2,5-dimethoxy-1,4-benzoquinones. Here, also the prenylated derivatives (31 and 32) showed higher efficiency and had similar inhibitory potential in cellular (IC_{50} 0.38 and 0.15 μM) as well as cell-free assays (IC_{50} 0.6 and 0.3 μM). The C16-alkyl-substituted compound 30 was poorly soluble and thus could not be tested for 5-LO inhibition. Taken together *ortho*-quinones with simple n-decyl, n-undecyl- or n-dodecyl-residues were most potent in intact human neutrophils with IC_{50} values in the submicromolar range (0.03 to 0.06 μM).

4.5.2 Selectivity of the benzoquinones for 5-LO compared to other LOs

Next, the influence of the compounds on platelet-type 12-LO and 12/15-LO (also termed 15-LO-1) was investigated after stimulation of platelets with AA or PMNL preparations with A23187 plus AA. With exception of compound 10 and 26 (both carrying a n-hexyl chain) none of the compounds (10 μM) inhibited the formation of 12-H(p)ETE from 12-LO and no compound the 15-H(p)ETE formation from 12/15-LO (Table 4.10).

Table 4.9: Effects of 4,5-methoxy-1,2-benzoquinones on inhibition of 5-LO.

No.	5-LO activity IC_{50} [μM] (remaining activity at 10 μM)		
	cell-based		cell-free
	A23187	A23187+AA	AA
25 R=	2.2± 0.8	5.1± 0.7	>10 μM (82.9± 2.7%)
26 R=	0.33± 0.1	0.99± 0.4	2.6± 0.3
27 R=	0.04± 0.01	0.07± 0.01	0.13± 0.01
28 R=	0.06± 0.01	0.10± 0.03	0.94± 0.4
29 R=	0.03± 0.01	0.15± 0.03	0.26± 0.02
30 R=	n.d.	n.d.	n.d.
31 R=	0.38± 0.1	0.39± 0.1	0.60± 0.1
32 R=	0.15± 0.07	0.10± 0.01	0.30± 0.1

n.d. = not determined

4.5.3 Inhibition of mPGES-1 and COX-1 by benzoquinones

Embelin was highly active against human mPGES-1 (compare 4.3.3, IC_{50} = 0.21 μM). Therefore, embelin-derived benzoquinones were tested for their ability to inhibit mPGES-1. As for isolated 5-LO, none of the tested compounds was superior to embelin on the inhibition of PGE_2 formation (Table 4.10). Only compounds 3, 5, 12, 13 and 21 showed inhibitory effects at mPGES-1 with IC_{50} values < 10 μM indicating that again the length of the alkyl chain in position 3 of the benzoquinone backbone is the determinant for the inhibitory potency. Lengths between 10 and 16 carbons appeared to be optimal for the inhibition. Within the different backbone groups, the 2,5-hydroxy-1,4-benzoquinones (3, 4, 5) were the most potent (IC_{50} values: 0.21 to 3.1 μM), followed by *O*-monomethylated derivatives (12, 13) (IC_{50} values: 9.1 and 5.8 μM, respectively) and *O,O'*-dimethylated compound 21 (IC_{50} value: 4.7 μM). *Ortho*-quinones however, did not inhibit PGE_2 formation for more than 50% up to 10 μM. Compound 28 and 29 were the most potent in this group inhibiting mPGES-1 by about 46% and 48%, respectively at 10 μM. The prenylated derivatives weakly inhibited PGE_2 formation. In this group, the 2,5-hydroxy-1,4-benzoquinones (7, 8) and *ortho*-quinones (31, 32) showed the highest potency. MK-886 was used as reference (inhibition 69% at 10 μM, reported IC_{50} = 2 μM (Koeberle et al., 2008a). Moreover, the effect of the compounds to interfere with COX-1 from platelets was investigated. Up to 10 μM only compounds 10, 20, 26, 27 and 31 inhibited 12-HHT formation for more than 40%. Interestingly, for compound 10 and 26 this inhibition parallels with the effect on platelet-type 12-LO suggesting a general effect on the platelets. Compound 10 did not show inhibition on 5-LO product formation

Table 4.10: Effects of benzoquinones on inhibition of 12-LO, 15-LO, COX-1 and mPGES-1.*Embelin

No.	12-HETE synthesis remaining activity at 10 μM AA	15-HETE synthesis remaining activity at 10 μM A23187+AA	COX-1 remaining activity at 10 μM AA	mPGES-1 IC_{50} [μM] or (remaining activity at 10 μM) PGH_2
1	93.3± 4.3%	82.3± 8.5%	81.4± 5.0%	(84.6± 2.0%)
2	105.3± 9.8%	89.8± 7.6%	96.8± 9.4%	(58.4± 9.1%)
3	76.0± 8.7%	90.0± 12.2%	81.1± 4.9%	1.1± 0.4
4*	74.9± 11.3%	91.5± 9.8%	89.5± 7.3%	0.21± 0.1
5	90.6± 3.1%	65.1± 19.5%	101.9± 10.4%	3.1± 0.4
6	117.1± 27.3%	104.7± 22.1%	122.5± 5.1%	(64.1± 9.9%)
7	80.5± 9.2%	92.0± 5.5%	129.6± 23.1%	(54.5± 2.9%)
8	78.3± 3.3%	95.2± 6.2%	85.4± 10.2%	(58.8± 5.6%)
9	91.2± 8.7%	111.6± 7.6%	102.6± 12.4%	(86.2± 9.8%)
10	44.5± 8.1%	65.8± 14.6%	55.1± 9.8%	(91.4± 0.2%)
11	122.0± 10.7%	122.7± 14.4%	68.1± 9.5%	(75.4± 9.9%)
12	138.4± 10.8%	131.2± 20.5%	80.5± 9.3%	9.1± 1.3
13	148.3± 19.7%	115.6± 4.5%	81.9± 5.4%	5.8± 0.1
14	137.5± 17.4%	118.9± 15.7%	88.4± 2.5%	(68.4± 1.4%)
15	100.8± 22.4%	111.5± 10.3%	129.6± 23.1%	(100.6± 7.4%)
16	107.6± 10.5%	122.1± 14.4%	85.9± 4.0%	(64.7± 5.8%)
17	102.6± 19.1%	103.1± 36.4%	93.0± 14.2%	(80.6± 13.2%)
18	101.5± 26.6%	100.8± 3.5%	64.2± 9.8%	(84.6± 3.4%)
19	143.5± 12.6%	n.d.	67.6± 5.0%	(77.5± 6.4%)
20	160.8± 36.1%	93.4± 16.8%	57.9± 4.5%	(59.2± 2.5%)
21	138.5± 18.3%	126.9± 5.5%	70.1± 5.9%	4.7± 0.6
22	121.0± 29.9%	97.8± 19.0%	91.6± 14.8%	(65.5± 4.3%)
23	90.3± 1.2%	126.8± 9.7%	89.4± 6.4%	(80.2± 6.9%)
24	138.7± 7.0%	135.7± 17.2%	78.5± 4.4%	(64.8± 4.3%)
25	85.8± 31.0%	128.8± 24.8%	92.4± 23.8%	(97.9± 2.9%)
26	18.6± 6.7%	91.7± 25.6%	24.4± 6.0%	(87.0± 1.8%)
27	133.1± 63.9%	147.8± 17.5%	42.9± 9.0%	(81.9± 6.9%)
28	235.8± 40.1%	104.7± 19.8%	62.2± 3.1%	(53.5± 11.6%)
29	134.8± 15.8%	132.9± 13.1%	87.8± 9.5%	(52.4± 2.6%)
30	n.d.	n.d.	n.d.	n.d.
31	72.8± 32.9%	124.6± 16.2%	47.2± 3.4%	(61.5± 2.3%)
32	211.0± 40.4%	174.3± 21.2%	75.3± 12.0%	(64.9± 5.7%)

n.d. = not determined

for more than 36% at 10 μM in cellular assays and 30% at the isolated enzyme. Also the conversion of PGH_2 to PGE_2 by mPGES-1 was not blocked efficiently. The inhibitory effect of compound 10 on 12-LO and COX-1 was therefore higher than for the other targets but still not highly efficient (about 60% inhibition at 10

μM). The *ortho*-quinones showed stronger interference with 12-HHT formation and 3 compounds (26, 27 and 31) inhibited 12-HHT formation by more than 40% at 10 μM. Compound 26 was active in PMNL stimulated with either A23187 or A23187 plus AA with IC_{50} values around 1 μM (A23187: 0.3 μM; A23187 plus AA: 1 μM). The inhibitory effect of compound 26 on 12-H(p)ETE and 12-HHT formation was therefore in a similar range (inhibition of about 80% at 10 μM). Compound 27 on the other hand was even more effective in blocking LT formation in human PMNL than compound 26 (IC_{50}: 0.04 μM, A23187 and 0.07 μM A23187 plus AA) and less active at COX-1 in platelets (inhibition by 58% at 10 μM). Assuming an IC_{50} value close to 10 μM for COX-1, compound 27 was more than 100-fold more efficient to supress LT than 12-HHT formation. Compound 31 was more potent on the inhibition of 5-LO product synthesis than platelet-derived 12-HHT formation, as well. In summary, the most active compound 3 against mPGES-1 (IC_{50} = 1.1 μM) was 5-fold less potent than embelin. Compared to 5-LO inhibition, the compounds were in general much less efficient at mPGES-1. As for 5-LO, chain lengths between 10 to 16 carbons were most efficient and prenylation did not improve potency at mPGES-1. Furthermore, compounds that showed to be highly active in cellular as well as isolated enzyme assays of 5-LO did not show interference with other cyclooxygenases and only some were also active at mPGES-1, however always to a weaker extend. On the other hand compounds 10 and 26 were more active on platelet-type 12-LO and COX-1 than on 5-LO.

4.5.4 Antioxidant ability of hydroxybenzoquinones

It is generally accepted that quinones are reduced in the intracellular environment in which high concentration of GSH (mmolar range) creates a reducing milieu. The formed "active" hydroquinones are able to scavenge radicals and therefore act as antioxidants (Ohkawa et al., 1991a). Since redox properties are also discussed as possible mechanisms for the inhibition of 5-LO by quinones in intact cells, the antioxidant and scavenging capacity of the compounds was assessed by a DPPH assay. Note that for embelin partial scavenging activities were described (Joshi et al., 2007). As shown in Fig. 4.40 the embelin (4) analogues (2,5-dihydroxy-1,4-benzoquinones, 1-3, 5-8) with two free hydroxy moieties scavenged the stable radical DPPH by about 40 to 50% independent of the 3-substituent. The *O,O'*-dimethylated 1,4-benzoquinones (17-22, 24) showed radical scavenging activities as well. However, O-monomethylated series (9-14, 16) and *ortho*-quinones (25-29, 32) failed to capture the DPPH radical. Only compound 15 and 24 showed weak radical scavenging activities. Nonetheless, compared to classical reducing agents like ascorbic acid and L-cysteine which were used as controls, the 1,4-benzoquinones were rather moderate scavengers. Especially the *ortho*-quinones which are most potent in 5-LO cellular assays failed in this respect. That shows, that other mechanism than simply redox or radical scavenging are responsible for the inhibition of 5-LO.
Next, the ability of the compounds to interfere with the formation of ROS in human PMNL was investigated. The ability to interfere with ROS formation in neutrophils did not strictly parallel the inhibitory impact observed for LT formation in neutrophils. A study of selected compounds revealed that the different benzoquinones backbones have differential effects on PMA-induced ROS formation in human PMNL. As shown in Fig. 4.41A among the compounds 5, 13, 21 and 29, only the 2,5-dihydroxy-1,4-benzoquinone inhibited ROS formation in PMNL at 10 μM while

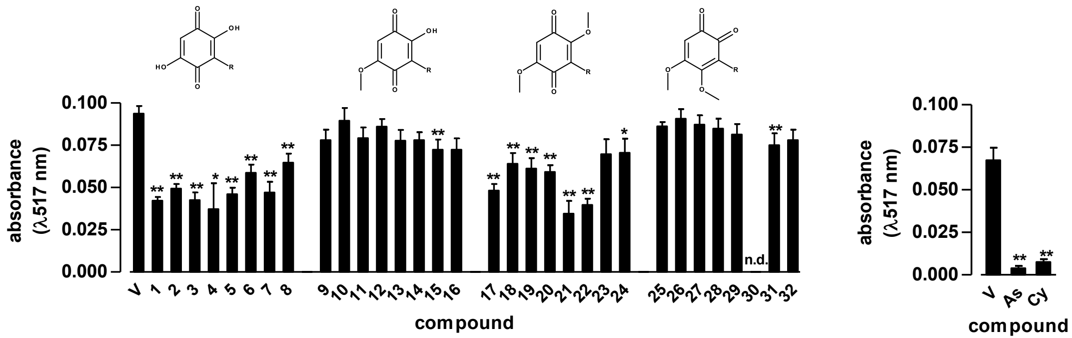

Figure 4.40: Effects of benzoquinones on DPPH scavenging abilities. Compounds (5 nmol) were incubated with 5 nmol DPPH for 30 min at RT and the absorbance was measured at 520 nm. Ascorbic acid and L-cysteine were used as controls. Values are given as absorbance at 517 nm, mean + S.E., n = 3. *,$p < 0.05$, **,$p < 0.01$ versus vehicle control (compounds: DMSO; ascorbic acid (AS), L-cysteine (Cy): H_2O).

others (compound 13 and 21) led to an increase in the formation of oxygen species. To a certain degree this was also evident in the unstimulated cells. Interestingly, compound 21 which was able to scavenge the DPPH radical did not reduce ROS formation in the cellular assay. Next, dihydroxybenzoquinone derivatives with varying chain lengths were analyzed (Fig. 4.41B). Increasing the chain length pronounced the inhibition of ROS formation with highest effects for 10 and 11 carbons (compound 3 and 4). The inhibition of ROS formation by dihydroxybenzoquinones correlates with the ability of compounds to scavenge the DPPH radical. *Ortho*-quinones tended to increase ROS formation in PMNL without further stimulation (Fig. 4.41C). However, after stimulation reduced oxygen species generation was observed for compounds 25, 27, 31 and 32 compared to vehicle control. Compound 29 was without effect. For the prenyl derivatives all possible backbones were analyzed. As shown in Fig. 4.41D, ortho quinones significantly reduced ROS formation while 2,5-dihydroxy-1,4-benzoquinones were less effective. Compound 23 was also active in this test system while the corresponding compound 24 was not. In summary, a differential effect on the inhibition of ROS formation is observed for linear and non-linear chains attached to the benzoquinone backbones (Fig. 4.41A and D). While derivatives with non-linear chains lead to a reduced ROS formation, corresponding compounds with linear chains did not interfere or even induced ROS formation in addition to PMA stimulation. Compounds with two free hydroxyl groups inhibit ROS formation and partially scavenge the DPPH radical as also observed for embelin. *O,O'*-dimethyl-1,4-benzoquinones did not interfere wtih ROS formation though partially scavenging DPPH. *Ortho*-quinones however, inhibited ROS formation without scavenging DPPH. Here, the length of the side chain between C10 and C11 caused the highest effect while 12 carbons showed no or smaller effects.

4.5.5 Inhibition of 5-LO product formation by benzoquinones in human blood

A blood assay was applied to test the compounds in an *in vivo* relevant environment. Upon preincubation of the compounds with human blood and subsequent stimulation with LPS/fMLP, aspects like protein binding or interference with fatty acids present in the blood can be studied. Thus, four derivatives with the optimal

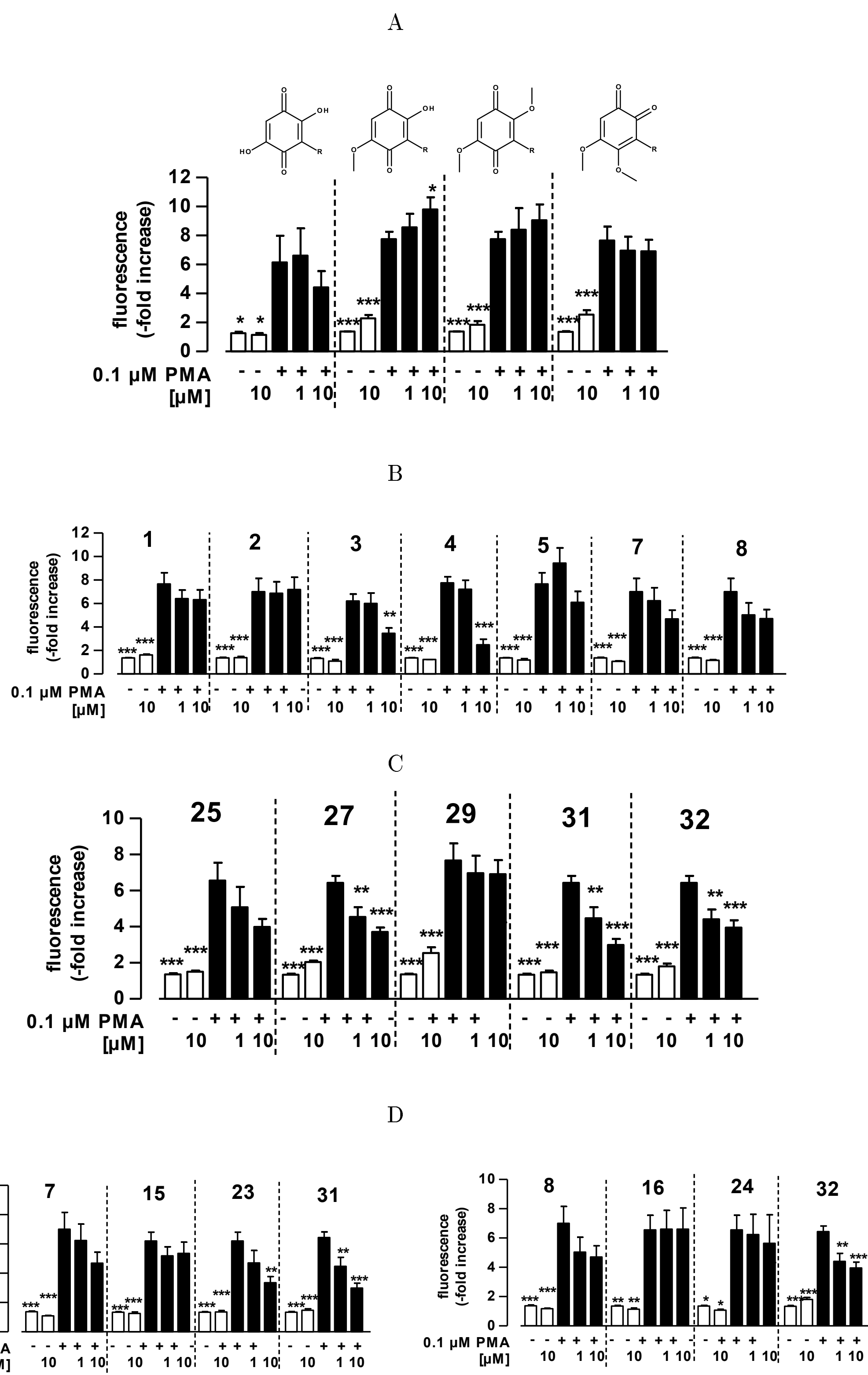

Figure 4.41: Influence of benzoquinones on ROS formation in human PMNL. PMNL were pre-incubated with compounds (or DMSO as vehicle control) for 15 min, loaded with the fluorescent dye DCF-DA and stimulated with 0.1 μM PMA. The relative increase in fluorescence was determined after at 37 °C after 360 s. Data (means + S.E.M., n = 3) are expressed as –fold increase (t = 0 (of unstimulated control) and t = 360 s). *,p < 0.05, **,p < 0.01, ***p < 0.001 versus stimulated vehicle control (A) Quinone derivatives from all backbone groups with a chain length of C12. (B) 2,5-dihydroxy-1,4-benzoquinones and (C) *ortho*-quinones (D) prenylated benzoquinone derivatives. For all experiments, 10 μM DPI was used as reference which inhibited ROS formation as expected (not shown). The experiments were performed by H. Traber (University of Jena).

chain length of 12 carbons in position 3 of the 4 different backbones were chosen. As shown in Fig. 4.42 the inhibitory efficiency in blood tremendously depended on the degree of methylation of the hydroxyl groups at the quinone ring. Embelin analogue 5 led to a concentration-dependent increase of LT formation in blood. The increase was measured for both LTB_4 and 5-H(p)ETE formation suggesting a stimulation of 5-LO product formation. Methylation of one hydroxyl group (compound 13) prevented the raise of product formation. However, no inhibition was observed up to 30 μM. Interestingly, double methylation of the hydroxyl groups gives compound 21 which potently inhibited 5-LO product formation in blood with IC_{50} around 3 μM. The corresponding *ortho*-quinone 29 was also active however to a lesser extent (IC_{50} 6.7 $\pm$ 1.3 μM). Compared to the cellular assays in which compound 21 and 29 inhibited LT formation with IC_{50} of 0.63 (0.49) and 0.03 (0.15) μM, respectively after stimulation with A23187 (A23187 plus AA) the inhibitory effect in blood was about 5 to 200 fold lower.

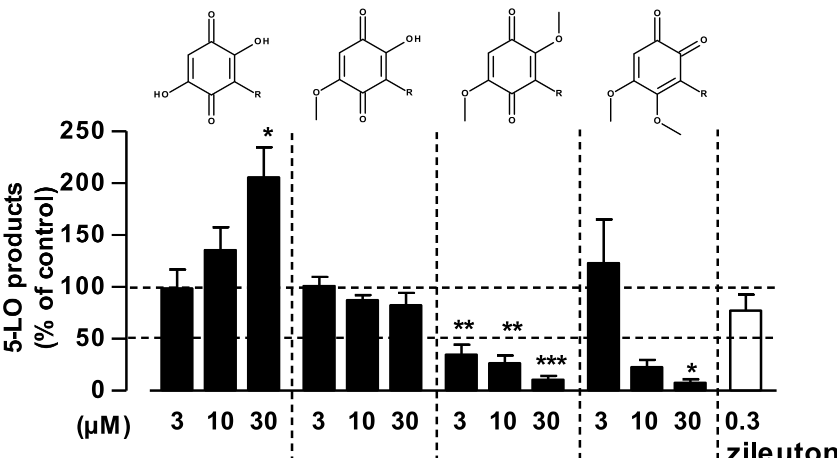

Figure 4.42: Inhibition of 5-LO product formation by benzoquinones in human whole blood. Human whole blood was primed for 15 min at 37 °C with 1 μg/mL LPS, treated with the test compounds (or DMSO as vehicle) for 15 min at 37 °C, and then stimulated with 1 μM fMLP. After 15 min at 37 °C, the formation of 5-LO products was determined (right panel). *,$p < 0.05$, **,$p < 0.01$, ***,$p < 0.001$ vs vehicle control. Data are means + SEM; n = 3-4.

4.5.6 Molecular pharmacological profile of 3-dodecyl-4,5-dimethoxy-1,2-benzoquinone

Since the *ortho*-quinone 3-dodecyl-4,5-dimethoxy-1,2-benzoquinone (compound 29) was the most potent benzoquinone derivative in cellular and purified enzyme assays it was chosen for further molecular pharmacological studies (Fig. 4.43). In human PMNL compound 29 inhibited 5-LO product synthesis with $IC_{50} = 0.03 \pm 0.01$ μM (A23187) and 0.15 ± 0.03 μM (A23187 plus AA) and at the isolated 5-LO enzyme with $IC_{50} = 0.26 \pm 0.02$ μM. Thus, there is a discrepancy in the efficiency in cell-free and cellular assays by 2- to 8-fold. For some inhibitors it is known that glycerides,

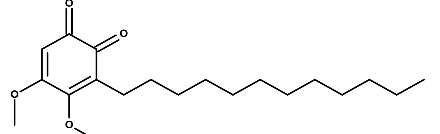

Figure 4.43: Chemical structure of 3-dodecyl-4,5-dimethoxy-1,2-benzoquinon (compound 29).

phospholipids or membranes interfere with the binding to and inhibition of 5-LO via the C2-like domain (i.e., hyperforin, (Feisst et al., 2009). However, evaluation of compound 29 revealed similar inhibitory curves for partially isolated 5-LO ($IC_{50} = 0.26 \pm 0.02$ μM) and homogenates of PMNL ($IC_{50} = 0.34 \pm 0.06$ μM) in which also cellular compartments such as proteins or lipids are present (Fig. 4.44A). Compound 29 interfered with 5-LO directly via reversible inhibition (Fig. 4.44B).

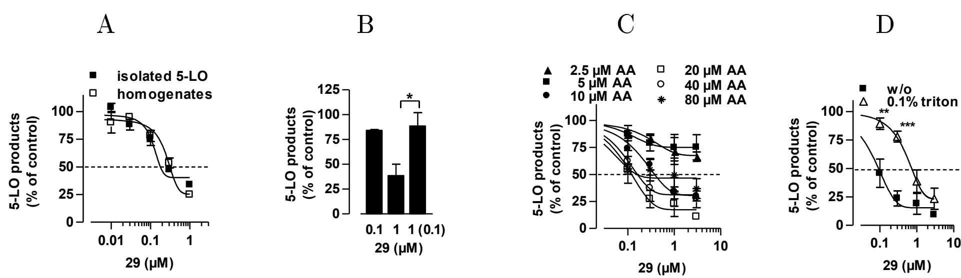

Figure 4.44: Inhibition of 5-LO by compound 29 in cell-free assays. (A) Partially purified recombinant 5-LO[a] or homogenates of PMNL (corresponding to 5×10^6 cells/ml) were incubated with compound 29 or vehicle (DMSO, 0.1%) at 4 °C for 15 min. Samples were prewarmed for 30 s at 37 °C, 2 mM $CaCl_2$ and 20 μM AA were added (10 min, 37 °C). Data are expressed as percentage of control (100%), means ± S.E.M., n = 3-5. (B) Reversibility of 5-LO inhibition by 29. Purified 5-LO was incubated with 0.1 μM or 1 μM 29 (15 min, 4 °C). An aliquot of the 1 μM sample was diluted with assay buffer 10-fold ("1 (0.1)"); the other aliquot was not altered. Then, samples were prewarmed for 30 s at 37 °C and 20 μM AA and 2 mM $CaCl_2$ (10 min, 37 °C). *, $p < 0.05$ versus inhibition without dilution. n = 3. (C) Partially purified 5-LO was incubated with compound 29 or vehicle (DMSO, 0.1%) (15 min, 4 °C) and stimulated with varying concentrations of substrate. Data are expressed as percentage of control (100%), means ±S.E.M, n = 3-5.[a] (D) Purified 5-LO was incubated with compound 29 in the absence or presence of 0.1% triton-X 100 and 5-LO activity was determined as mentioned above. **,$p < 0.01$ versus inhibition without addition of triton. Data are expressed as percentage of control (100%), means ±S.E.M, n = 3-4. [a]The assays were performed by H. Traber (University of Jena).

Due to the fatty acid-like structure of the dodecyl-chain in position 3, a competitive binding mode might be possible. Interestingly, as shown in Fig. 4.44C lower concentration of substrate AA (2.5 and 5 μM) did not lead to significant inhibition while increase in AA up to 20 μM AA enhanced the inhibitory potential. At higher

concentrations of 40 and 80 μM AA the inhibitory effect was again lower than at 20 μM AA. Therefore, the inhibitory efficacy followed a hyperbolic shape with an optimum at 20 μM AA. At 5 μM AA isolated 5-LO was only inhibited up to 22% at 3 μM. The calculated IC_{50} values were 0.41 ± 0.1 μM (10 μM AA), 0.18 ± 0.1 μM (20 μM), 0.93 ± 0.3 μM (40 μM), 2.99 ± 1.6 μM (80 μM). No IC_{50} could be estimated for 2.5 and 5 μM AA. As mentioned before, inhibitors might be sensitive to the addition of non-ionic detergents in the incubation preparations. As shown in Fig. 4.44D compound 29 was less active if triton was added. The IC_{50} shifted by about 7-fold from 0.13 ± 0.1 μM to 0.97 ± 0.4 μM (with 0.1% triton). In order to further underline the potent inhibitory potential of compound 29, the compound was tested in A23187-stimulated monocytes (Fig. 4.45A). In human monocytes the IC_{50} value was about two fold higher than in human PMNL but still in the nanomolar range (IC_{50} = 69 ± 27 nM, monocytes and IC_{50} = 29 ± 10 nM, neutrophils). Via the addition of OAG to cell preparations interactions with the C2-like domain of the 5-LO enzyme can be studied. However, as shown in Fig. 4.45B and C the inhibitory efficiency of compound 29 was not essentially affected by the addition of OAG to either PMNL (Fig. 4.45B) or monocytes (Fig. 4.45C).

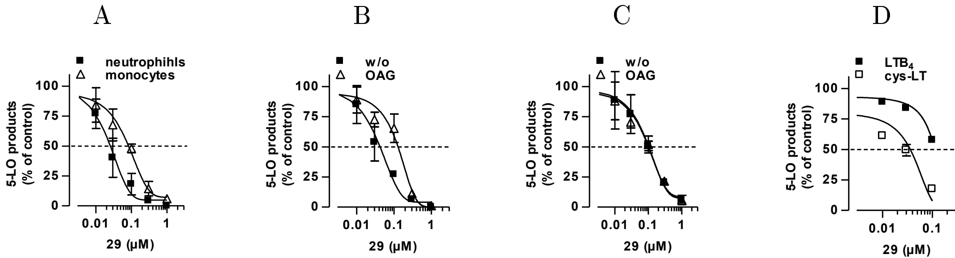

Figure 4.45: Effect of compound 29 on LT formation in neutrophils and monocytes. (A) Cells (10^6/ml) were stimulated with 2.5 μM A23187 after preincubation with compound 29 for 15 min at 37 °C. Data are expressed as percentage of control (100%), means ± S.E.M., n = 3. (B, C) PMNL (B) and monocytes (C) (10^6/ml) were preincubated with compound 29 followed by incubation with 30 μM OAG for 3 min prior stimulation with 2.5 μM A23187. Data are expressed as percentage of control (100%), means ± S.E.M., n = 2. (D) Human monocytes were primed with 1 μg/ml LPS for 5 min at 37 °C, and incubated with compound 29 or vehicle (0.1% DMSO) for 15 min at 37 °C and stimulated with 1 μM fMLP for 10 min at 37 °C. Data are expressed as percentage of control (100%), means ± SEM., n = 3. Cys-LT was determined with EIA and LTB_4 with HPLC-UV.

The inhibitory potential of compound 29 was tested in a more pathophysiological relevant assay system to evaluate the inhibitory capacity on cys-LT formation. Thus, human monocytes were primed with LPS followed by fMLP and formed cys-LT measured by EIA. Compound 29 potently inhibited cys-LT formation in monocytes with higher potency than LTB_4 formation (IC_{50} = 22 ± 2 nM) (Fig. 4.45D). Next, interference of compound 29 with the translocation of 5-LO from the cytosol to the nuclear membranes was investigated. The different cell fractions were generated by mild lysis with detergent NP-40 (0.1%). As shown in Fig. 4.46 compound 29 did not prevent nor potentiate the distribution of 5-LO between nuclear and non-nuclear membranes. Hyperforin which was used as control hindered the translocation of 5-LO to nuclear membranes as expected.

In the cell, the release of the substrate AA by $cPLA_2$ is a critical step in the synthesis of LTs. Inhibition of $cPLA_2$ leads to the reduction of product formation by downstream enzymes. Compound 29 did not influence the release of AA from cell-free

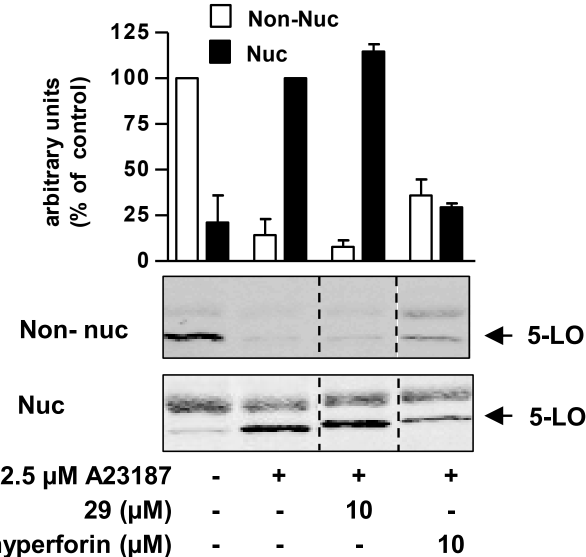

Figure 4.46: Influence of compound 29 on subcellular distribution of 5-LO. Human PMNL were preincubated with 10 μM hyperforin, 10 μM 29 or vehicle (0.1% DMSO) for 15 min at 37 °C and stimulated with 2.5 μM A23187 for 5 min at 37 °C. 5-LO was monitored by Western Blot of 5-LO in the nuclear (Nuc) and non-nuclear (Non-nuc) fractions of mild-detergent (0.1% NP-40)-lysed cells. Results shown are representatives of 3 independent experiments. Dashed lines show where the membranes were cut to take out additional samples. Data are expressed as percentage of control (the 5-LO band from unstimulated PMNL was set to 100% in Non-nuc, the 5-LO band from A23187-activated PMNL was set 100% in Nuc.; means + SEM. n = 3.

$cPLA_2$ or in monocytes stimulated with A23187 (Table 4.11). In order to further elucidate which additional factors might be responsible for the shift of IC_{50} between isolated enzyme and cellular assays towards lower values, phosphorylation of ERK was assessed. However, compound 29 did not inhibit fMLP-induced phosphorylation of ERK (Fig. 4.47A). The increase of intracellular Ca^{2+} following stimulation of PMNL and monocytes leads to activation of $cPLA_2$ and 5-LO. Ca^{2+} favors the localization of 5-LO at the nuclear membranes and therefore leads to its activation. Compound 29 did not interfere with the increase of intracellular Ca^{2+} following stimulation with fMLP (Fig. 4.47B).

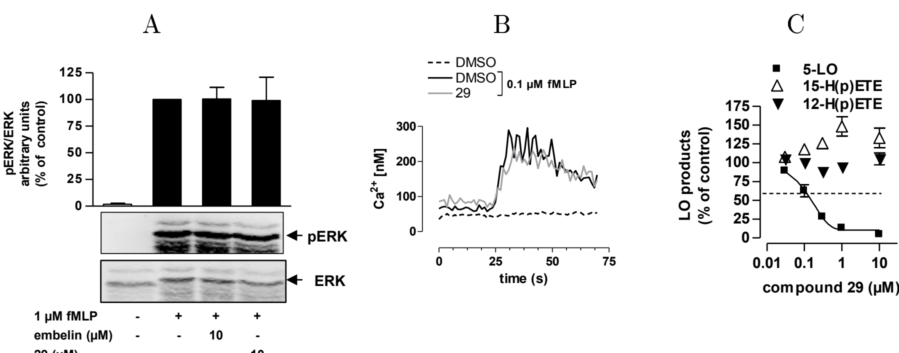

Figure 4.47: Influence of compound 29 and embelin on ERK phosphorylation, Ca^{2+}-mobilization and 12- and 15-LO. (A) Human PMNL from female donors were preincubated with compounds or vehicle control (0.1% DMSO) for 15 min at 37 °C followed by stimulation with 1 μM fMLP for 1.5 min at 37 °C. One representative of 2 independent experiments is shown. (B) Fura-2/AM loaded human PMNL were preincubated with compound 29 at 10 μM for 15 min at 37 °C and the baseline recorded for 20 sec. The increase of intracellular Ca^{2+} was by 0.1 μM fMLP. Data are means of nM Ca^{2+} from 3 independent experiments. (C) Cells (5×10^6/ml) were stimulated with 2.5 μM A23187 plus 20 μM AA. Data are expressed as percentage of control (100%), means $\pm$ S.E.M., n = 3-7.

Compound 29 selectively inhibited 5-LO product formation in human PMNL stimulated with A23187 plus AA. Under these conditions no inhibition of 5-LO related 15-LO from eosinophils or platelet-type 12-LO from PMNL-adhering platelets was observed (Fig. 4.45C). Additionally, further LO related targets were investigated. As shown in Table 4.11 compound 29 inhibited the conversion of PGH_2 to PGE_2 by microsomal preparations from IL-1β-stimulated A549 cells by 48% at 10 μM. However, in a cell-based assay of LPS-stimulated human monocytes and in the blood assay the formation of PGE_2 was not reduced (Table 4.11). The reference inhibitors blocked PGE_2 formation as expected. Furthermore, the effect of compound 29 on cyclooxygenases (COX-1 and 2) was tested in cellular assays, in which 29 displayed the highest efficiency on the inhibition of LT formation. No inhibition was observed on 12-HHT formation in platelets (COX-1) or on the 6-keto $PGF_{1\alpha}$ formation in LPS stimulated monocytes (COX-2). In summary, compound 29 is a selective inhibitor for 5-LO, considering enzymes of the eicosanoid pathway.
After 20 h incubation, compound 29 showed some cytotoxic effects at 10 μM on primary monocytes while no significant effects were observed for cancer cell line A549 even after longer incubation periods. For short-time incubations (30 min), conditions resembling the LT assays, trypan blue exclusion was assessed without any effects on the viability of PMNL (Fig. 4.48). In control cells treated with DMSO (0.1%) viability was 94.7 $\pm$ 0.12% and in cell treated with 29 93.6 $\pm$ 0.8% (n = 2). Therefore, one can exclude that cytotoxic effects lead to the inhibition of LT formation observed. This is further supported by the fact that no inhibition of other related target enzymes is obvious.

Table 4.11: Effects of compound 29 on AA release, PGE_2 formation and the activity of COX-1 and 2. Compound 29 (10 μM) or reference inhibitors (at the indicated concentrations) were added to the respective enzymes, blood or freshly isolated human monocytes or platelets 15 min prior induction of the reaction. Data (means ± S.E., n = 3) are expressed as percentage of the remaining activity of the uninhibited vehicle (0.1% DMSO) control (100%). indo = indometacin, cele = celecoxib. [a]The experiments were executed by M. Melzer, University of Jena.

enzyme/ assay	compound 29 (10 μM) % remaining activity or IC_{50}	reference control % remaining activity
$cPLA_2$, cell-free[a]	98.3 ± 8.2%	24.2 ± 4.9% (RSC-3388, 5 μM)
^{3}[H]AA release from monocytes	99.8 ± 0.9%	16.6 ± 8.5% (RSC-3388, 5 μM)
LPS stimulated monocytes (PGE_2)	101.5 ± 5.2%	22.2 ± 4.7% (MD 52, 5 μM) 9.1 ± 1.7% (indo, 10 μM) 21.8 ± 1.0% (cele, 5 μM)
LPS stimulated blood (PGE_2)	95.9 ± 19.6%	16.3 ± 7.3% (indo, 50 μM)
COX-1 platelets	87.8 ± 9.5%	22.4 ± 9.6% (indo, 10 μM)
COX-2 (6-keto $PGF_{1\alpha}$ formation from LPS stimulated monocytes)	78.4 ± 5.6%	32.6 ± 8.0% (cele, 5 μM)

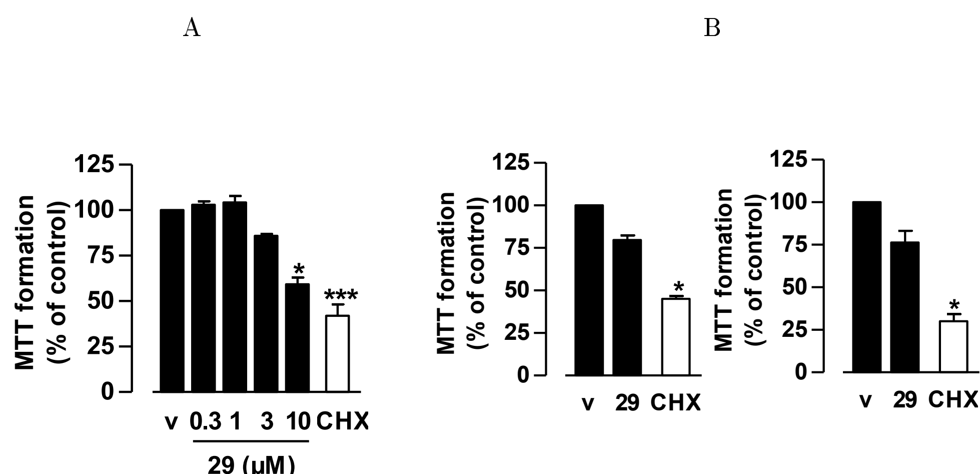

Figure 4.48: Effect of compound 29 on cell viability. (A) Effect of compound 29 (10 μM) on LPS stimulated monocytes, (B) A549 after 24 h preincubation (48 h for B, right panel) the formation of MTT was measured after cell lysis by absorption at 595 nm. 50 μM cycloheximid (CHX) was used as reference control. Data are expressed as percentage of control (100%), means + SEM, n = 3. *, $p < 0.05$; **, $p < 0.01$ versus vehicle control (v = DMSO 0.3%).

As already shown in Fig. 4.12 compound 29 inhibited 5-LO product formation in blood after stimulation with pathophysiological relevant stimuli (LPS/fMLP: IC_{50} 6.7 ± 1.3 μM). As shown in Fig. 4.49A compound 29 also interfered with the for-

mation of LTs following stimulation with 30 μM A23187 (IC_{50} = 10.3 ± 3.5 μM). 12-H(p)ETE and 12-HHT formation were not reduced under these conditions. However, compound 29 was not superior over the control inhibitor zileuton (Fig. 4.49B). Zileuton was about 10-fold more potent (LPS/fMLP: IC_{50} = 0.9 ± 0.4 μM, A23187: 0.9 ± 0.1 μM). Taken together, compound 29 potently inhibited LT formation in cellular and blood assays. On the molecular level, no further target other than 5-LO was identified that was responsible for the potentiation of inhibitory activity in the cells compared to isolated 5-LO enzyme.

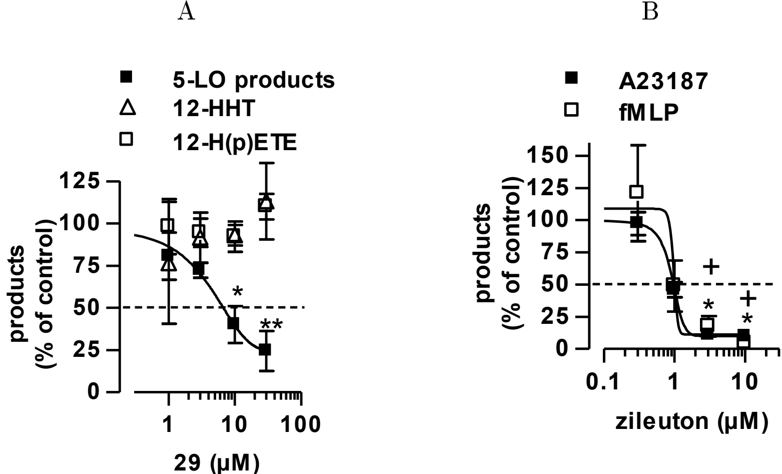

Figure 4.49: Effect of compound 29 on LO and COX product formation in blood. (A, B) Human blood was preincubated with compounds or vehicle control (0.1% DMSO) (15 min, 37 °C). Product formation was induced by 30 μM A23187 (10 min, 37 °C). Data are expressed as percentage of DMSO control (100%) means ± SEM., n = 3-4. *, $p < 0.05$ vs vehicle control. (B) Human blood was primed with 1 μg/ml LPS (30 min, 37 °C) and preincubated with zileuton or vehicle control (0.1% DMSO) (15 min, 37 °C). LT formation was induced by the 0.1 μM fMLP (15 min, 37 °C). Data are expressed as percentage of DMSO control (100%) means ± SEM, n = 3. *, $p < 0.05$ versus vehicle control (A23187), +, $p < 0.05$ versus vehicle control (fMLP).

5 Discussion

LTs are involved in the pathophysiology of autoimmune diseases such as asthma, allergic rhinitis and cardiovascular diseases as well as cancer (as reviewed in (Haeggström et al., 2010)). Besides, a sex-bias is observed for autoimmune diseases and one underlying mechanism might be the divergent abilities to form LTs by males and females (Whitacre, 2001; Pergola et al., 2008, 2011). This bias is dependent on the male sex hormone testosterone, which leads to reduced LT formation in blood, and in isolated neutrophils and monocytes from males compared to females (Pergola et al., 2008, 2011). In view of this difference, the question was raised if (and how) pregnancy influences LT formation in females. Pregnancy is accompanied by major changes within the immune system, a consistent rise of sex hormones as well as altered incidences of immune diseases (Veenstra van Nieuwenhoven et al., 2003). First, the impact of pregnancy and menstrual cycle on LT formation will be discussed. Then discovery of potent new inhibitors of 5-LO, their SARs and their molecular mode of action will be addressed. Furthermore, the plausibility of benzoquinones as redox-type 5-LO inhibitors will be commented.

5.1 Pregnancy influences LT formation

Human blood was applied as test system to study the impact of pregnancy on LT formation since isolated cells in suspension behave differently from cells studied in their physiologic environment, that is, the plasma (Veenstra van Nieuwenhoven et al., 2003). LT formation was higher in blood from pregnant compared to non-pregnant females applying various stimuli. This upregulation is composite of three effects: (I) higher numbers of LT forming cells, (II) lower LT formation capacity of isolated granulocytes and (III) upregulating effects of plasma from pregnant donors on LT formation from isolated enzyme and cells. In summary, higher LT synthesis in blood of pregnant females is influenced by synergistic and opposite effects. The effect of pregnancy on LT formation in peripheral blood has not been investigated before. However, lower 5-LO product formation in isolated neutrophils from pregnant compared to non-pregnant donors was reported, which is consistent with the results presented in this thesis (Imai and Arai, 1996; Crocker et al., 1999). Detailed studies on the underlying mechanisms have not been conducted so far. The amount of catalytically available 5-LO enzyme, intracellular mobility of 5-LO, Ca^{2+}-mobilization, MAPK phosphorylation, intracellular redox tone and provision of substrate AA might be altered in cells from pregnant females resulting in lower LT biosynthesis. To compare the amounts of catalytically 5-LO enzyme in granulocytes, several assays were applied. Cells were stimulated with excessive amounts of AA as substrate, and 5-LO enzyme activity as well as enzyme expression was investigated in cell homogenates. No significant differences between samples from pregnant and non-pregnant females were found. Therefore, it can be concluded that the differences in LT formation capacity do not result from significantly different amounts of catalytically 5-LO enzyme in cells from pregnant and non-pregnant do-

nors. Other mechanisms, such as reduced supply of substrate or cellular activation of 5-LO, may be responsible. While it was reported that AA release in granulocytes from pregnant donors was reduced compared to cells from non-pregnant females (Crocker et al., 1999), preliminary results in this thesis show that AA release was not different following stimulation with A23187. Another feature that was reported and also addressed in this thesis is the lower ability of granulocytes to form ROS (Kindzelskii et al., 2002, 2004). Interestingly, reduced ROS formation parallels with lower LT formation in neutrophils from pregnant donors. Parallels between pregnancy and sepsis were suggested (Sacks et al., 1998). *Ex vivo* stimulation of neutrophils from patients suffering from sepsis revealed lower LT and ROS formation as well as delayed apoptosis compared to healthy controls (Mayer et al., 2003; Wesche et al., 2005). Addition of AA, which elevates the oxidative state of the cells (Carrillo et al., 2011), minimized the difference between pregnant and non-pregnant female derived granulocytes in regard of LT formation. However, attempts to show that hydroperoxides (i.e. 13-HPODE) can substitute for lower ROS formation in cells from pregnant donors failed in this respect. Likewise, inhibition of NADPH oxidase by DPI did not support the hypothesis that LT formation in cells from pregnant females depends on the availability of ROS. Investigations in neutrophils from male and female donors revealed that LT formation in cells from males is reduced due to basal ERK activation by testosterone and consequent nuclear localization of 5-LO (Pergola et al., 2008). Those mechanisms were not investigated in this study but might be responsible for the reduced formation of LTs in isolated granulocytes from pregnant donors. It was suggested by others that altered levels of circulating fatty acids might lead to lower responsiveness of innate immune cells during pregnancy (Crocker et al., 1999). Besides ROS, neutrophil chemotaxis (Bjorksten et al., 1978), bacterial killing (Bjorksten et al., 1978) and adherence (Krause et al., 1987) were described to be reduced during pregnancy. Therefore, the lower ability to form LTs by cells from pregnant donors emphasizes the observation that peripheral cells of the innate immune system are somewhat inactivated. Activation of neutrophils by plasma might lead to reduced responsiveness following subsequent stimulation. This might be compensated by an increase in neutrophil and monocyte cell number leading to enhanced LT formation in blood. Delayed apoptosis of neutrophils seems to be responsible for the neutrophilia observed throughout pregnancy (von Dadelszen et al., 1999).

During pregnancy several plasma components are significantly modulated (Carlin and Alfirevic, 2008). Lower LTB_4 values were reported in serum from pregnant donors during 2nd trimester (Jian et al., 2013). To analyze the effect of plasma on LT formation, purified 5-LO was resuspended in plasma from pregnant and non-pregnant females. In these experiments a direct effect on the 5-LO enzyme was observed and plasma from pregnant donors led to higher LT formation compared to non-pregnant females. Furthermore, plasma from pregnant females activates LT formation in neutrophils and also partially in monocytes (plasma from 2nd trimester) compared to plasma from non-pregnant females following stimulation with A23187. This implies an effect on the cellular level. The release of AA was not differentially influenced by plasma from pregnant compared to non-pregnant donors. Therefore, effects at the level of $cPLA_2$ by the plasma can be excluded. Exogenous supplementation of AA reduced these differences to equal levels of LT formation, which suggests that this excessive stimulation might overcome the relatively weak stimulatory factor in plasma from pregnant females. Preliminary experiments showed

that resuspension of cells from pregnant women in autologous plasma led to higher amounts of products than the combination of plasma from pregnant females and cells from non-pregnant women. This suggests further interaction of cells from pregnant females in their physiologic environment which are not found for granulocytes of non-pregnant women. Presence of stimulatory or absence of inhibitory factors in the plasma from pregnant women might be responsible for the observed effects. The nature of these factors was not clarified in this thesis but could be of endocrine, metabolic or even fetal origin. Proteins like MIF, leptin, HCG and sex hormones in physiological relevant concentrations did not increase LT formation in cells related to the effects observed by plasma. Testosterone was only slightly increased in the course of pregnancy and a detrimental effect on LT formation was described for testosterone (Pergola et al., 2008). Similarly, estradiol and progesterone did not increase LT formation in monocytes as shown in this thesis and in neutrophils (data not shown) though modulation of the immune system was described for female sex hormones (Osorio et al., 2008; Hughes et al., 2013). It is unlikely that sex hormones are responsible for the observed effects by the plasma from pregnant donors.
So far it is unclear, if the higher LT formation during pregnancy is part of the modulation of the immune system during pregnancy or a consequence of the ongoing changes. However, the results ask for a reevaluation of the treatment of asthma with LTRAs during pregnancy.

5.2 LT and eicosanoid biosynthesis are altered in the course of menstrual cycle

An auxiliary finding in this thesis is the observation that the menstrual cycle impacts LT and eicosanoid biosynthesis. In stimulated blood from non-pregnant females 5-LO derived products were lower whereas 12-HHT formation was higher in samples drawn during follicular phase compared to samples from luteal phase. Platelet counts were higher in samples from females during follicular phase compared to luteal phase. But no correlation between platelet count and 12-HHT formation capacity of the blood was found. These results are best confirmed by longitudinal studies accompanied by sex hormone measurements and exact estimations of the cycle phase. However, physiological implications and pathophysiological courses of LT related diseases support the opposing synthesis capacities of respective products. A higher risk for acute myocardial infarction in early follicular phase was reported (Mukamal et al., 2002). However, detailed analysis of the activation state of platelets and platelet-leukocyte aggregate formation are controversial. While some reports found no influence of menstrual cycle, there are indications for platelet-leukocyte activation around ovulation (Robb et al., 2010; Rosin et al., 2006). Platelet aggregation induced by diverse stimuli was reported to be lowest in mid-luteal phase (Melamed et al., 2010). Interestingly, the influence of exercise on platelet activation was only observed during follicular phase (Wang et al., 1997). These observations underline the higher 12-HHT formation capacity of stimulated blood derived from females during follicular phase. 5-LO products were higher in samples from female donors during luteal phase compared to follicular phase. It is known that the course of autoimmune diseases is influenced by the menstrual cycle (Oertelt-Prigione, 2012). Premenstrual asthma is characterized by poor disease control, aspirin sensitivity and associated with severe exacerbations (Rao et al., 2013). Treatment of premenstrual

asthma with cys-LT receptor antagonists has been shown to be beneficial (Nakasato et al., 1999; Pasaoglu et al., 2008). Interestingly, serum levels of LTC_4 were elevated during asthma exacerbations in patients suffering from premenstrual asthma (Nakasato et al., 1999). However, some studies neglect the benefit of cys-LT receptor antagonists and no changes of LTC_4 levels were reported (Pereira-Vega et al., 2012). Besides, LTs were related to the occurrence of dysmenorrhea (Abu and Konje, 2000). Interestingly, elevated urinary LTE_4 levels were reported at the beginning of menstruation accompanied by dysmenorrhea but not in case of eumenorrhea (Harel et al., 2000). While in some studies treatment of dysmenorrhea with cys-LT receptor antagonists was beneficial (Fujiwara et al., 2010), it was not in others (Harel et al., 2004). While exercise increases platelet activation during follicular phase, exercise during luteal phase induces higher amount of total gene regulations (Wang et al., 1997; Northoff et al., 2008). Upregulation of pro-inflammatory genes was higher than their downregulation or the upregulation of anti-inflammatory genes. Interestingly, the genes for 5-LO (5-ALOX) and $CysLT_1$ were upregulated in luteal phase and downregulated in follicular phase following exercise (Northoff et al., 2008). The observation that LT levels in stimulated blood from females during luteal phase are higher compared to follicular phase supports the involvement of LTs in premenstrual asthma and dysmenorrhea. Attempts to identify the underlying mechanisms for higher 12-HHT and lower LT formation during follicular phase, by analysis of cell numbers, product formation in isolated cells or impact of plasma derived from follicular or luteal phase, did not lead to explanations. Intercellular interactions, for example between leukocytes and platelets, might be possible. Stimulation with Ca^{2+} mobilizing agents unspecifically activates both, neutrophils and platelets. Interestingly, interaction between platelets and neutrophils via fibrinogen and integrins (GPIIb/GPIIIa and CD11b/CD18) lowered 5-LO product formation by neutrophils (Chabannes et al., 2003a,b). Similarly, interaction of neutrophils and platelets via P-selectin glycoprotein ligand-1 and P-selectin, 12-HHT and 12-HETE formation was reduced in platelets (Chabannes et al., 1994). Therefore, it might be possible that neutrophils and platelets interact in blood differently depending on the time of the menstrual cycle.

5.3 The mechanism of 5-LO inhibition by benzoquinones

The molecular mechanism of how 1,4-benzoquinones inhibit 5-LO activity is not clearly addressed in literature and precise evaluations are missing so far. The results of this thesis question the hypothesis that benzoquinones inhibit 5-LO solely via a redox-type or ligand binding fashion. If activated into the corresponding hydroquinone (e.g. in intact cells or in presence of reducing agents) they may act as reducing agents by uncoupling the redox cycle of the active site iron in 5-LO by their radical scavenging features (Ohkawa et al., 1991b) (Fig. 5.1). As a consequence, benzoquinones are usually more active in cellular compared to cell-free assays. However, it was shown that certain 1,4-benzoquinones which potently inhibit 5-LO exhibit poor antioxidant and radical scavenging activities (Czapski et al., 2012). Finally, antioxidant abilities and 5-LO inhibition did not correlate in a study with various 1,4-benzoquinones (Wurm and Schwandt, 2003). Indeed, some 1,4-benzoquinones such as embelin are highly active in cell-free assays in which reducing agents are

not present (Filosa et al., 2013; Schaible et al., 2013b). Since under these assay conditions higher amounts of the quinone form are present, reduction of the iron and disruption of the redox cycle are unlikely. The mode of 5-LO inhibition by dihydroxy-1,4-benzoquinones is mainly dependent on the structure of the residues attached to the quinone backbone. It was suggested that benzoquinones compete with AA as substrate for binding to 5-LO (Yoshimoto et al., 1982). AA-861, for example, indeed showed competition against AA (Hofmann et al., 2012). Moreover, the lipophilicity of the compounds parallel with their potencies. This is consistent with the results derived from a study addressing benzoquinones decorated with residues of varying polarity (Filosa et al., 2013). As examplified by embelin potent interaction with the 5-LO enzyme is possible irrespective of the redox form of the inhibitor and interference with the binding of AA without necessarily competing it, is suggested. On the other hand, as described for the structurally related compound RF-Id, the reduced hydroquinone form shows higher activity than the oxidated quinone. A nonredox-type fashion might be an underlying fashion since the postulated binding site by docking studies revealed a higher number of bidirectional hydrogen bonds by the hydroquinone form. However, after bioactivation into the respective hydroquinone, also a redox-type inhibition might be possible for RF-Id as depicted in Fig. 5.1. Since the quinone backbone is the same between embelin and RF-Id it becomes evident that the modification of the adjacent residues determines the mode of inhibition. Moreover, embelin and RF-Id were specific in regard to the inhibition of 5-LO. 12- and 15-LO, which are related enzymes with a catalytic redox cycle, which is even more sensitive to disturbances, were not inhibited. In summary, this thesis poses new questions in respect of the mechanism of 5-LO inhibition by benzoquinones and shows that this is not necessarily a redox-type fashion.

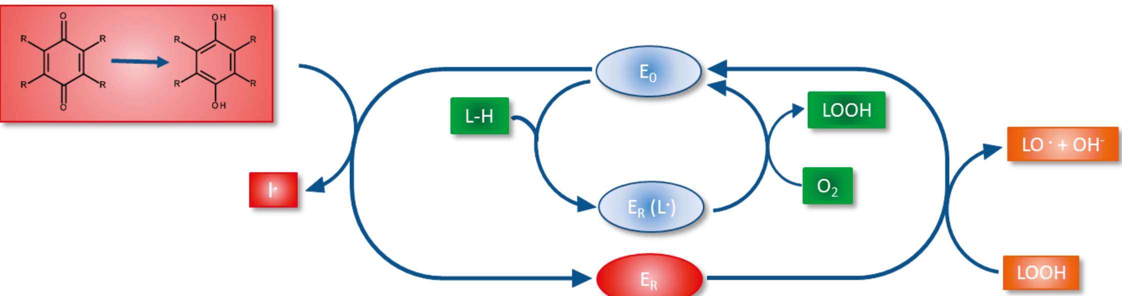

Figure 5.1: Traditionally proposed interference of benzoquinones with 5-LO. Reduction of 1,4-benzoquinone into hydroquinone and interruption of the catalytic cycle of 5-LO. E_0 = oxidized, active 5-LO; E_r = reduced, inactive form of 5-LO; LOOH = fatty acid hydroperoxide; L-H = substrate; I = redox-type inhibitor. The inner cycle represents the catalysis of 5-LO and its substrate AA. The right part of the outer cycle illustrate the oxidative activation of 5-LO by lipid hydroperoxides. The left part shows the reductive inactivation of 5-LO by redox-type inhibitors.

5.4 Potent inhibition of 5-lipoxygenase and microsomal prostaglandin synthase-1 by embelin

5-lipoxygenase and mPGES-1 were revealed as direct molecular targets of embelin with IC_{50} values at the isolated enzymes of 0.06 and 0.2 μM, respectively. Embelin

inhibited isolated 5-LO more potent than zileuton (IC_{50} = 0.6 μM), the only 5-LO inhibitor on the market (Carter et al., 1991). In addition, embelin was superior to related 1,4-benzoquinone AA-861 (Yoshimoto et al., 1982) and naturally occurring benzoquinones such as aethiopinone, ardisianone A, ardisiaquinones, measanin and thymoquinone (IC_{50} values: 0.11-1 μM) ((Werz, 2007) and references therein). Similary, embelin inhibits mPGES-1 more potently than other naturally derived inhibitors (myrtucommulone, garcinol, arzanol, curcumin, β-boswellic acid, and epigallocathechin gallate) with IC_{50} values $>$ 0.3 μM ((Koeberle and Werz, 2009) and references therein). Dual action of 5-LO inhibitors on mPGES-1 was described for several compounds such as triazole-based carboxylic acids (De Simone et al., 2011), α-alkyl-substituted pirinixic acid derivatives (Koeberle et al., 2008a), acylphloroglucinols (hyperforin, garcinol, arzanol, myrtucommulone) (Koeberle and Werz, 2009), indomethacin and lonazolac derivatives (Elkady et al., 2012) and arylpyrrolizines (Liedtke et al., 2009). mPGES-1 inhibition was concentration-dependent in presence of Triton X-100, which indicates that embelin is not a nuisance inhibitor as it was described for other inhibitors (Wiegard et al., 2012). The specific inhibition was only partially reversible upon wash-out and independent of the PGH_2 substrate concentration. Defined molecular interactions were not addressed in this thesis. However, docking experiments suggest a concrete binding site for embelin in the central pore of the homotrimeric protein and the region adjacent to the GSH binding site. While the n-undecanyl chain fills the central pore, the quinone ring groups are involved in hydrogen bounds to GSH as well as to Tyr-117 and Arg-70. Those two amino acids are residues of the central pore. The binding of embelin might therefore interfere with the same route the substrate PGH_2 uses to reach the catalytic site (Jegerschold et al., 2008). Selectively interacting with 5-LO and mPGES-1, embelin did not significantly affect related enzymes such as 12- and 15-LO, $cPLA_2$, COX-1 or COX-2. Dual 5-LO/mPGES-1 inhibitors often fail to inhibit COX enzymes (Koeberle and Werz, 2009; Elkady et al., 2012). Recently, interaction of embelin with Tyr-355 of COX-2 was suggested in a silico analysis. But biological testing was not performed (Landa et al., 2011). Further molecular pharmacological evaluation excluded FLAP and ERK as targets while ROS formation was reduced by embelin. This might be an additional mechanism for the interference with LT formation.

Characterization of the mode of 5-LO inhibition of embelin

Embelin inhibited LT formation in PMNL and monocytes applying various stimuli (A23187, A23187 plus AA, LPS/fMLP), the potency of embelin in cell-free assays was up to 20-30 fold higher. The loss of inhibitory potency in cellular assays might be related to the benzoquinone ring of embelin with its vinylog acids. Due to its fatty acid-like structure, uptake as well as distribution within the cell might be hindered. In contrast to the antioxidants ascorbic acid and L-cysteine, embelin barely reduced the DPPH radical as described before (Joshi et al., 2007; Mahendran et al., 2011b). In the cell-free 5-LO activity assay no reducing agent (e.g. thiol) was present that might have converted embelin to the hydroquinone form, but still embelin potently inhibited 5-LO. In fact, inclusion of DTT into cell homogenate assays did not improve the inhibitory potency while this was the case for the 1,4-benzoquinone AA-861. Therefore, it can be assumed that embelin inhibits 5-LO in the quinone form. Note that embelin did not inhibit the structurally related 12-LO or 15-LO that convert the 1,4-pentadiene structure of AA by a similar mechanism as 5-LO and are thus also susceptible to changes of the redox cycle of the active-site iron. Hence, a different

molecular mechanism than simply uncoupling the redox cycle of the active site iron is obvious. Embelin exhibits two dissociable protons. At a pH range of 6.0 to 8.0, which is present under physiological conditions in the extracellular space and in the cytosol of intact cells, embelin is monodeprotonated (Rani et al., 2010). In this way, relations of embelin to the chemical structure of fatty acids are obvious and access into 5-LO via the substrate binding channel is conceivable. Fatty acids and fatty acid-like molecules are known to modulate 5-LO catalytic activity (Werz, 2007). Docking studies proposed distinct binding sites for embelin at the catalytic domain of 5-LO. The n-undecanyl chain of embelin fills the hydrophobic channel where the oxidation of AA takes place in the uninhibited 5-LO enzyme (Skrzypczak-Jankun et al., 2001). Stabilization of the positioning is formed by the benzoquinone as well as the hydroquinone form with Gln-363, Gln-557 and Tyr-181. Interestingly, Tyr-181 seems to play an important role in the opening mechanism of 5-LO for AA as substrate (Gilbert et al., 2011, 2012). However, in a study with the 5-LO Tyr181Ala mutant the importance of the interaction between embelin and Tyr-181 to inhibit 5-LO could not be proven. Embelin equally interfered with LT formation at wild-type and mutant 5-LO. Competition with AA was described underlying 5-LO inhibition by AA-861 (Yoshimoto et al., 1982; Hofmann et al., 2012). This was not observed in respect of embelin. Therefore, purely displacing AA from the active site is excluded as inhibitory mechanism. Embelin might also act as an iron ligand inhibitor like zileuton. Indeed, the formation of binary complexes between embelin and divalent metal cations like Co(II), Ni(II), Cu(II) and Zn (II) via an oxo moiety and the neighboring hydroxyl group are described (Rani et al., 2010). However, the docking studies do not support chelating interactions of the iron by embelin. Electron spin resonance studies should be performed to further clarify this issue. The binding of embelin to 5-LO was completely reversible upon wash-out, therefore covalent interaction or irreversible chemical reactivity with the 5-LO enzyme can be excluded. This was described for U73122 or α-tocopherol (Hornig et al., 2012; Reddanna et al., 1985). Embelin inhibited equally well in purified enzyme preparations as well as in neutrophil homogenates indicating that cellular components such as phospholipids are not involved in the inhibition. Hyperforin and pyridinylimidazol EP-6 interact with the C2-like domain of 5-LO and their inhibitory efficiency was reduced in cell homogenates versus purified 5-LO and phospholipids counteracted their potency (Reddy et al., 2000; Feisst et al., 2009; Wisniewska et al., 2012). Interference with the biosynthesis of pro-inflammatory mediators LT and PGE_2 might be of biological relevance with regard to the biological effects of embelin described for *in vivo* animal models. Interestingly, several targets for embelin were described so far. Embelin interfered with proliferation and was pro-apoptotic in various cancer cells (see (Kim et al., 2013) and references therein). Of note, the IC_{50} value for XIAP antagonism in a cell-free binding assay for embelin was 4.1 μM and in prostate cancer cells PC-3 and LNCaP highly expressing XIAP apoptosis was induced with IC_{50} values of 3.7 and 5.7 μM, respectively (Nikolovska-Coleska et al., 2004). Embelin inhibited NFκB activation at 30 μM (Reuter et al., 2010), which is apparently mediated by $PPAR_\gamma$ and STAT-3 (at 15-50 μM) (Dai et al., 2009; Heo et al., 2011). Though the inhibitory efficiency on 5-LO is lower in cellular assays, IC_{50} values are still in the micromolar range and superior (at least 20-fold at isolated proteins) to other targets that were described. However, the *in vivo* relevance of the interference of embelin with 5-LO and mPGES-1 has not been determined so far. Dual inhibitors of 5-LO and mPGES-1 are being discussed as potential candidates for cancer therapy (Koeberle and Werz,

2009; Rådmark and Samuelsson, 2010). Therefore, the interaction of embelin with 5-LO and mPGES-1 represents new targets which might explain the observed *in vivo* effects of embelin in models of inflammation and cancer in addition to already described targets.

5.5 Compound RF-Id inhibits 5-LO not via redox-type but rather by a nonredox type fashion

Similar to embelin, compound RF-Id inhibited 5-LO product formation in neutrophils and monocytes with equal potencies upon various cell stimulation conditions. Moreover, RF-Id blocked LT formation in human blood (IC_{50} = 4.1 μM) and possesses anti-inflammatory efficacy *in vivo*. Many potent inhibitors fail in blood assays due to strong albumin-binding, interaction with blood cells or plasma components such as enzymes or small molecules (vitamins, metal ions, lipids) (Pergola and Werz, 2010). Therefore, these results of RF-Id in whole blood are encouraging. Though interaction with the 5-LO pathway might explain *in vivo* efficiency, other targets such as inflammatory cytokine release and NFκB signaling as described for atrovirinone may be suggested (Syahida et al., 2006; Israf et al., 2010). Furthermore, the used animal models are not strictly LT-dependent. However, recent results show that in exudates of zymosan-elicited mice upon air pouch LTB_4 levels were reduced but not PGE_2 levels after treatment with RF-Id (not shown). In cellular and cell-free assays, RF-Id only inhibited cellular COX-2 activity but no other related enzyme within the eicosanoid synthetic pathway ($cPLA_2$, COX-1, mPGES-1, 12/15-LO) was affected. Therefore, RF-Id inhibited 5-LO as well as COX-2 selectively. Interestingly, RF-Id was significantly less potent at isolated 5-LO compared to cellular assays. This is a common feature observed for compounds interfering with the 5-LO activation pathway. MK-886 and related compounds are targeting FLAP and thereby inhibit LT formation by 5-LO only in the cellular context (Dixon et al., 1990). Besides this, tryptanthrin and pirinixic acid derivatives potently inhibit 5-LO product formation in cellular assays but only moderately interact with 5-LO itself (Pergola et al., 2012; Greiner et al., 2011). Compound RF-Id inhibited equally well in preparations of cell homogenates and isolated 5-LO excluding that cellular components like membranes are responsible for the inhibitory activity. FLAP interaction and $cPLA_2$ inhibition were excluded by specific assays such as 5-LO translocation and variation of exogenous substrate AA concentrations as well as $cPLA_2$ activity assays. However, reducing conditions established by addition of DTT to cell homogenates or isolated enzyme preparations increased the inhibitory potential of RF-Id. Accordingly, applying the thiol oxidizing agent diamide in cellular assays, which elevates the oxidative tone in neutrophils, counteracted the potency of RF-Id. Interestingly, similar behavior is observed for structurally different nonredox-type inhibitors (i.e., ZM230487, L-739,010, CJ-13,610) (Werz et al., 1998; Fischer et al., 2004). Nonredox-type inhibitors compete with fatty acid hydroperoxides and a similar mechanism might be possible for RF-Id (Werz et al., 1998). Docking studies with RF-Id show that the reduced hydroquinone form is able to interact via bidirectional hydrogen bonds to a higher extent as the quinone core. This further supports the idea that RF-Id is activated in the cellular environment by reduction and in the following interacts with 5-LO in a nonredox-type fashion. Recent inhibitor studies with the hydroqui-

none form of RF-Id supported this hypothesis (Data by Verena Kraut, University of Jena, Germany, not shown). The described bioactivation also represents a drawback of RF-Id since at inflammatory sites usually an oxidative environment is found. However, the promising results from *in vivo* studies still make RF-Id an interesting candidate for further drug research.

5.6 Structure-activity relationships of benzoquinones

Encouraged by the potent ability of RF-Id and embelin to interfere with LT formation, benzoquinones were designed to evaluate SARs. Due to the need of reducing conditions for the activity of RF-Id in cells, which counteracts the oxidative environment at inflammation sites, structural variations were continued based on embelin in an attempt to increase the inhibitory efficiency in cells. As depicted in Fig. 5.2 differential SARs are found for the interaction with 5-LO in cell-free as well as cellular environment by the benzoquinones. While embelin potently inhibited 5-LO

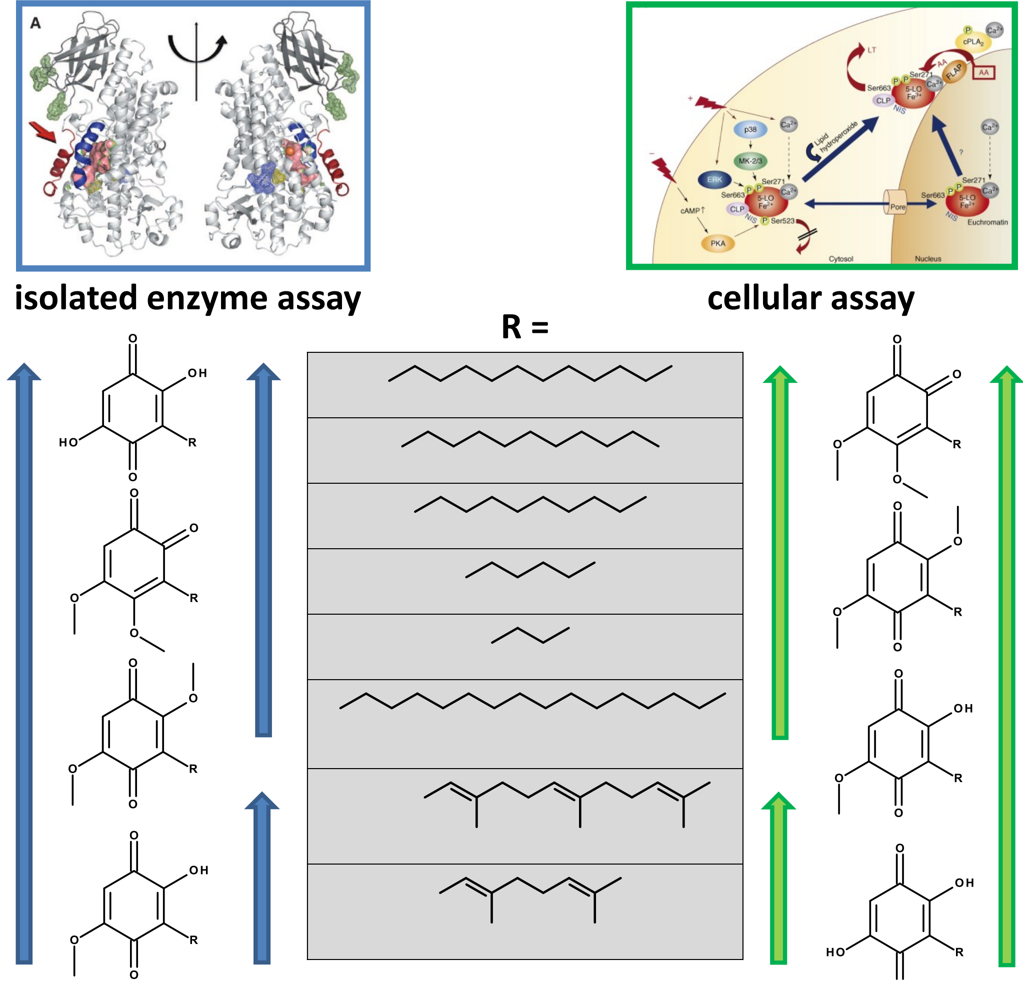

Figure 5.2: Summary of the SARs for the inhibition of hydroxybenzoquinones on 5-LO. Arrows indicated increasing inhibitory capacity on 5-LO product formation. Illustrations were taken from (Gilbert et al., 2011) and (Rådmark et al., 2007).

directly, its potency was lower in the cellular context. Hence by step-wise methylation of the hydroxyl residues at the quinone core, efficiency in cellular and blood assays was achieved. Due to the observation that the lipophilicity and the structure of the residue attached to the quinone backbone discriminates the mode and potency of inhibition, the length of the alkyl chain was varied. Interestingly, the created compounds resemble lipid fatty acids and competition with AA might be possible. Indeed, comparing the inhibitory efficiency in cellular assays slight loss of inhibition was found stimulating with substrate AA together with A23187 compared to A23187 alone. Compounds decorated with a 2,5-dihydroxy-1,4-benzoquinone backbone inhibited isolated 5-LO with IC_{50} values between 0.17 and 4 μM, but lost efficiency in cellular assays similarly to embelin ($IC_{50} > 1.7$ μM). Due to the amphiphilic nature of the 2,5-dihydroxy-1,4-benzoquinone backbone, which incorporates two vinylog acids with dissociable protons and a lipophilic chain, the penetration of cellular membranes of the compounds might be hampered and therefore be responsible for the dropping activity in cellular assays. This disadvantage was overcome by methylation of one or two hydroxyl groups and compounds were highly active in cellular assays (IC_{50} values between 0.2 and 4.1 μM, stimulation with A23187). However, the efficacy to inhibit isolated 5-LO dropped following methylation especially for compounds with alkyl chains of 6 or less carbon atoms. *O,O'*-dimethylated analogues exhibited 2- to 3-fold higher activity at the isolated enzyme than corresponding monomethylated derivatives. Interestingly, by restructuring the quinone core to create 4,5-methoxy-1,2-benzoquinones (*ortho*-quinone structures) substantial progress was made and even higher efficiency for 5-LO inhibition gained in cellular and cell-free environment. The potency against the isolated enzyme resembled that of embelin-related compounds. In neutrophils the inhibition was even stronger than at the isolated enzyme and IC_{50} values for the most potent compounds were in the nanomolar range (0.03 to 0.06 μM). The higher potency in cells might originate either from the involvement of additional targets, from intracellular enrichment or from bioactivation. Besides the quinone core, the alkyl residue is crucial for the inhibitory activity of the compounds. Generally, alkyl chains of 10, 11 and 12 carbon atoms are most potent within their backbone group. Elongation to C16 was detrimental and resulted either in low solubility or poor inhibitory efficiency. Similarly, the prenyl chain with three isoprene moieties was superior to two. Two or three prenyl residues connected to 1,4-hydroquinone were found before to be superior in suppression of LTB_4 synthesis in neutrophils as compared to one or four moieties (Terencio et al., 1998). The prenyl derivatives exhibited lower potency compared to the compounds with linear chains. Compounds with optimal chain lengths are fatty acid-like and therefore interaction with 5-LO in the catalytic site of the enzyme seems to be likely. The hydrophobic chain can easily fill the channel for substrate AA. It is surprising that the described compounds interact rather specifically with 5-LO and only a few also target 12- and 15-LO and COX-1. Interestingly, it was observed that the shorter the chain, the more unspecific inhibition occurred. Especially compounds with 6 carbons were more prone to unspecific reactions. In addition, the 5-LO specificity is contradictory to the hypothesis that benzoquinones are redox-type inhibitors as discussed for embelin and RF-Id. This observation indicates the presence of specific binding sites within the catalytic center of 5-LO. Furthermore, compared to 5-LO inhibition only moderate activity was found at mPGES-1. Compounds decorated with vinylogic acids were superior to other benzoquinones and within this group the optimal chain length was represented by embelin (11 carbon atoms).

As described for embelin, 2,5-dihydroxy-1,4-benzoquinones partially scavenged DPPH as expected. This parallels the reduction of ROS formation observed in the cellular assay. Here, the effect was higher for compounds with 10 and 11 carbon atoms. *Ortho*-quinones interfered with ROS formation though they did not scavenge DPPH. Therefore, direct interaction with ROS generating enzymes might be possible and may be one reason for the higher intracellular activity of *ortho*-quinones. The effect was dependent on the chain length and prenyl residues were superior to linear. It is surprising that dimethylated benzoquinones partially scavenge DPPH. However, certain instability of the compounds in ethanolic solutions and partial reduction to the hydroquinone form may account for the observed phenomenon. The suitability for further *in vivo* studies was investigated in a blood assay with a group of four compounds with the optimal chain length of 12 carbon atoms. The behavior in blood depended on the methylation grade of the hydroxyl groups. The 2,5-dihydroxy-1,4-benzoquinone even led to increased product formation, which might be explained by competition with fatty acids bound to plasma proteins. Through this AA, which is available in plasma to certain amount, might be liberated and could be converted to LTs by 5-LO. Single methylation hinders this action, but no inhibition of LT formation was observed. The compound may unspecifically interfere with plasma components. The strongest inhibition was observed for *para*-quinone with an IC_{50} below 3 μM, which is encouraging for further studies. *Ortho*-quinone similarly was able to inhibit LT formation in blood but to a lesser extent although its activity in cellular assays was more than 10-fold higher compared to the *para*-quinone.

Molecular pharmacological profile of the 3-dodecyl-4,5-dimethoxy-1,2-benzoquinone

To further characterize the molecular pharmacological profile of *ortho*-quinones, the most potent benzoquinone, that is, compound 29 (3-dodecyl-4,5-dimethoxy-1,2-benzoquinone) was selected. The inhibitory potency was in the range of highly active nonredox-type 5-LO or FLAP inhibitors (neutrophils, A23187 stimulation; 29: IC_{50} = 0.03 μM; CJ-13,610: IC_{50} = 0.07 μM; ZM230487: IC_{50} = 0.02 μM, MK-886: IC_{50} = 0.03 μM (Fischer et al., 2004, 2003; Gillard et al., 1989). Rare information is available on the inhibition of 5-LO by *ortho*-quinones. Aethiopinone derived from *Salvia aethiopsis* inhibits 5-LO in neutrophils as well as the isolated enzyme with an IC_{50} of 0.2 μM and 0.11 μM, respectively (Benrezzouk et al., 2001). The potent cellular activity of compound 29 was further underlined applying different cell types (neutrophils and monocytes) and different stimuli (LPS/fMLP, A23187, A23187 plus AA) as well as diverse product readouts (LTB_4 and cysLT). In all assays, 29 potently interfered with LT formation. Even higher potency was observed for cysLT formation in LPS/fMLP stimulated monocytes (IC_{50} = 0.02 μM). However, direct interference of 29 with purified 5-LO was 2- to 8-fold lower compared to cellular activity. Several factors that are known to increase cellular inhibitory potency were analyzed such as modulation by glycerides, phospholipids or membranes, inhibition of $cPLA_2$, interference with 5-LO translocation, modulation of ERK phosphorylation and of Ca^{2+}-mobilization. No influence of 29 in either assay was noted. Possibly, the cellular trapping of the compound by, for example, cleavage of the methyl bonds increases the intracellular concentration and the inhibitory activity. Direct 5-LO inhibition of compound 29 was reversible upon wash-out. Interestingly, poor inhibition was observed at low substrate AA concentrations (2.5 and 5 μM AA) and high AA amounts (80 μM) were again detrimental. This phenomenon was also observed for

HZ-52, a pirinix acid derivative (Greiner et al., 2011). This suggests that a second putative AA binding site is involved in the activity of 5-LO at high AA concentrations (Aharony and Stein, 1986) might be inhibited by 29. However, it is also likely that high AA amounts are needed to form micelles that incorporate 29, which is then more efficiently presented to 5-LO (Greiner et al., 2011). The experiment in which triton X-100 lowered the potency of 29 might explain the latter. Triton X-100 might intervene with AA micelle formation and thereof lower incorporation of 29. Compound 29 inhibited 5-LO selectivly without interfering with related enzymes such as 12-/15-LO, COX-1/2, $cPLA_2$ or mPGES-1. Cytotoxicity was not the cause of the potent inhibition of LT synthesis since over the incubation period of the 5-LO assay compound 29 did not reduce cellular vitality.
In summary, structural modifications of benzoquinones lead to potent 5-LO inhibitors, such as the *ortho*-quinone compound 29 with promising activity in cells and in blood valuable for future pharmacological studies.

6 Conclusions

Within this thesis it is shown that during pregnancy LT formation in blood is increased. This adds further knowledge to the field of immune regulation during pregnancy on the side of innate immune system. Furthermore, by the attempt to elucidate this upregulation, a concept of three factors either acting in synergism or in opposite directions was elaborated. The described data are in coherence with published results concerning the role of LTs in parturition. It is still unclear to which extent higher LT formation is important for the maternal immune system. However, the results clearly ask for a reevaluation of the application of LTARs for asthma treatment of pregnant asthmatic patients.

The second part dealing with benzoquinones as exogenous modulators of 5-LO identified the natural compound embelin as potent inhibitor of 5-LO and mPGES-1. With this, new targets of embelin are presented for which lower concentrations are needed for inhibition than for other described targets. Besides embelin, also RF-Id and the *ortho*-quinone 29 were studied in cell-free, cellular and blood assays for their interference with LT synthesis and identified as potent 5-LO inhibitors. Mechanistic studies showed that embelin and RF-Id, though similarly structured, interfered with 5-LO in different modes. Interestingly, both inhibitors do not inhibit via redoxtype 5-LO inhibition as often supposed for benzoquinones in literature. This shows that detailed mechanistic studies are important to understand the interaction of inhibitor and enzyme. From SAR studies of benzoquinones the group of *ortho*-quinones came up as promising chemotype that has not been studied in respect of 5-LO inhibition in detail until now. However, whether or not the presented compounds are of pharmacological relevance remains to be studied.

List of Publications

Original publications (included in this thesis)

Filosa, R.; Peduto, A.*; Aparoy, P.*; Schaible, A.M.*, Luderer, S.; Krauth, V.; Petronzi, C.; Massa, A.; de Rosa, M.; Reddanna, P.; et al., Discovery and biological evaluation of novel 1,4-benzoquinone and related resorcinol derivatives that inhibit 5-lipoxygenase. Eur J Med Chem 2013, 67C, 269-279. * authors contributed equally

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Filosa, R.; Schaible, A.M.; Krauth, V. ; Tarber, H.; Weinigel, C.; Barz, D.; Werz, O.; Discovery and SAR of embelin derived benzoquinones that inhibit 5-lipoxygenase and microsomal prostaglandin E_2 synthase-1. In preparation.

Schaible, A.M.; Filosa, R.; Tarber, H.; Dehm, F.; Luuer, S.; Weinigel, C.; Barz, D.; Werz, O.; The inhibition of 5-lipoxygenase by an orthobenzoquinone and its molecular pharmacology in vitro and in vivo. In preparation.

Original publications (not included in this thesis)

Richter, P.; Schubert, G.; Schaible A.M.; Levent, C.; Werz, O.; and Pohnert, G. (2014). Caulerpenyne and Related Bis-enol Esters Are Novel-Type Inhibitors of Human 5-Lipoxygenase. ChemMedChem. In press.

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Posterpresentations

Rogge, A.; Pergola, C.; Werz, O.; Influence of pregnancy on leukotriene formation. 3rd European Workshop on Lipid Mediators, Pasteur Institute - Paris, June 3-4, 2010

Rogge, A.; Pergola, C.; Werz, O.; Differential eicosanoid biosynthesis during the menstrual cycle. Joint meeting of the "Austrian and German Pharmaceutical Societies", Innsbruck, September 20-23, 2011

Pergola, C.; Rogge, A.; Dehm, F.; Rossi, A.; Sautebin, L.; Werz, O.; Leukotriene biosynthesis is sex-biased in human monocytes. Joint meeting of the "Austrian and German Pharmaceutical Societies", Innsbruck, September 20-23, 2011

Oral presentations

Rogge, A.; Pergola, C.; Werz, O.; Influence of pregnancy on leukotriene formation: A prime example for personalized medicine. Jahrestagung der Deutschen Pharmazeutischen Gesellschaft e.V., Braunschweig, October 4-7, 2010

Rogge, A.; Pergola, C.; Werz, O.; Analyse von Leukotrienen als entzündlich-allergische Mediatoren in der Schwangerschaft. Invited Talk. VWR Chromforum Berlin, May 10-11, 2011

Schaible, A.; Pergola, C.; Werz, O.; Analyse von Leukotrienen als entzündlich-allergische Mediatoren in der Schwangerschaft. Invited Talk. VWR Chromforum Hamburg, February 28-29, 2012

Schaible, A.; Pergola, C.; Werz, O.; Analyse von Leukotrienen als entzündlich-allergische Mediatoren in der Schwangerschaft. Invited Talk. VWR Chromforum Halle, June 5-6, 2012

Schaible, A.; Pergola, C.; Werz, O.; Leukotriene biosynthesis during pregnancy. Summerschool of FIRST (Frankfurt International Research Graduate School for Translational Biomedicine), Löwenstein, August 29-31, 2012

Schaible, A..; Pergola, C.; Werz, O.; Analyse von Leukotrienen als entzündlich-allergische Mediatoren in der Schwangerschaft. Invited Talk. VWR Chromforum Darmstadt, November 5, 2012

Schaible, A.; Traber, H.; Filosa, R.; Weinigel, C.; Barz, D.; Werz, O.; Potent inhibition of human 5-lipoxygenase and microsomal prostaglandin E2 synthase-1 by the anti-carcinogenic and anti-inflammatory agent embelin. Doktorandentagung der Deutschen Pharmazeutischen Gesellschaft, Weimar, November 14-17, 2012

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Selbstständigkeitserklärung

Hiermit erkläre ich, dass mir die Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller Universität in Jena bekannt ist. Ich habe die Arbeit selbst angefertigt, keine Textabschnitte eines Dritten oder eigener Prüfungsarbeiten ohne Kennzeichnung übernommen und habe alle benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit gekennzeichnet und angegeben. Bei der Auswahl und der Auswertung des Materials, sowie bei der Herstellung der Manuskripte hat mich Prof. Dr. Oliver Werz unterstützt.

Ich versichere, dass ich die Hilfe eines Promotionsberaters nicht in Anspruch genommen habe und kein Dritter mittelbar oder unmittelbar geldwerte Leistungen für Arbeiten erhalten hat, die in Verbindung mit der vorliegenden Arbeit stehen. Die vorliegende Arbeit wurde von mir bei keiner bisherigen Prüfungsarbeit für staatliche oder wissenschaftliche Prüfungen eingereicht. Weiterhin versichere ich, dass ich gleiche, in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

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