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Identification and characterization of sulindac sulfide as a novel type of 5-lipoxygenase inhibitor with clinical relevance

Dissertation

Identification and characterization of sulindac sulfide as a novel type of 5-lipoxygenase inhibitor with clinical relevance

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Meinen Eltern

Das schönste Glück des denkenden Menschen ist, das Erforschliche erforscht zu haben und das Unerforschliche zu verehren.

Johann Wolfgang von Goethe

Contents

Abbreviations

12-HHT	12(S)-hydroxy-5-cis-8,10-trans-heptadecatrienoic acid
2xLB	2 x loading buffer
5-HETE	5-(S)-hydroxy-6-trans-8,11,14-cis-eicosatetraenoic-acid
5-LO $^{-/-}$ mouse	5-LO double knock-out mouse
5-LO-3W	triple W 5-LO mutant
5-LO-wt	wildtype 5-LO
5-Oxo-ETE	5-Oxo-eicosatetraenoicacid
5xLB	5 x loading buffer
A23187	calcium ionophore or ionomycin
AA	arachidonic acid
Ala	alanine, amino acid
Alox-5	arachidonate 5-lipoxygenase
ASA	aspirin, acetylic salicylic acid
BWA4C	non chelating 5-LO inhibitor
cAMP	cyclic adenosine monophosphate
ChIP	chromatin immunoprecipitation assay
CLP	coactosin-like protein
CML	chronic myeloic leukemia
COX	cyclooxygenase
cPLA2	cytosolic phosphilipase A_2
cys-LTs	cysteinyl leukotrienes
DAG	diacylglycerol

DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetate
ERK 1/2	p42/44 MAP-kinase or extracellular regulated kinase
FLAP	5-lipoxygenase activating protein
fMLP	N-formyl-methionyl-leucyl-phenylalanine
GPCR	G-protein coupled receptor
GPx	glutathione peroxidase
HDAC	histone deacetylases
HeLa	cervix carcinoma cell line
HPLC	high pressure liquid chromatography
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
LT	leukotriene
LTB_4	leukotriene B_4
LTC_4	leukotriene C_4
LTD_4	leukotriene D_4
LTE_4	leukotriene E_4
MAPEG	membrane-associated proteins in eicosanoid and glutathione metabolism
MAPK	mitogen-activated protein kinase
MGST2,3	microsomal glutathione S-transferase-2, 3
MK-886	FLAP inhibitor
MM6	Mono Mac 6
MS-275	Histone deacytelase (HDAC) inhibitor

NF-kB	nuclear factor kappa ß
NO-ASA	NO-aspirin, NO-acetylic salicylic acid
NSAID	non-steroidal antiinflammatory drug
NP-40	detergent
OAG	1-oleyl-2acetyl-*sn*-glycerol
PA	phosphatidic acid
PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	prostaglandin
PGC buffer	PBS containing glucose and $CaCl_2$
PGE_2	prostaglandin E_2
PIP	posphatidylinositol-bisphosphate
PMNL	polymorphonuclear leukocytes
PMSF	phenylmethanesulfonylfluoride
PPARα	peroxisome proliferator-activated receptor
PS	phosphatidylserine
ROS	reactive oxygen species
S100	100.000xg supernatant
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ssi	sulindac sulfide
Sso	sulindac sulfone
STI	soybean-trypsin-inhibitor
Sul	sulindac

TFA	trifluoroacetic acid
TGF-ß	transforming growth factor beta
Tris	tris(hydroxymethyl)aminomethane
Trp	tryptophan, aromatic amino acid
ZD-2138	non-redox type 5-LO inhibitor
Zil	zileuton, iron-ligand 5-LO inhibitor

1 INTRODUCTION

1.1 Non-steroidal anti-inflammatory drugs

1.1.1 Classification of NSAIDs

Non-steroidal anti-inflammatory drugs (NSAIDs) display a wide spread group of drugs used to treat a variety of diseases related to inflammatory and painful processes in acute and chronic conditions. Several compounds are on today's drug market differing in their therapeutic potential and mainly in their adverse side effect profiles. The origin of anti-inflammatory drugs was found in the salicylates, a familly of carboxylic acids, discovered in the middle of the 19th century were aspirin® becomes the progenitor of the NSAIDs. Followed by the development of phenylbutazone and later indomethacin, more drugs reached the market and ibuprofen was the first NSAID available without prescription. Classification of the drugs is typically carried out by means of their chemical structures and properties. Today, therefore still the carboxylic acids exist, including subgroups like the salicylates, the fenamates, the indole/indene actetates, the phenylacetates and the propionates**.** Another group display the oxicames with piroxicam and meloxicam as prominent agents (refer to Fig.1) [1]. High efforts were made to gain more knowledge about the molecular mechanisms of NSAIDs and in may 1999, the COX-2 selective inhibitor rofecoxib, was released to the market displaying the first drug of a novel group of NSAIDs. The coxibes, which are indicated for treatment of rheumatoid arthritis, osteo-arthritis and other arthritic diseases, dental and surgical pain in postoperative seetings and acute injuries were developed [2]. Structural variations of coxibs followed, but unfortunately rofecoxib had to be withdrawn by Merck from the market only a short period later due to severe cardiovascular side effects [3].

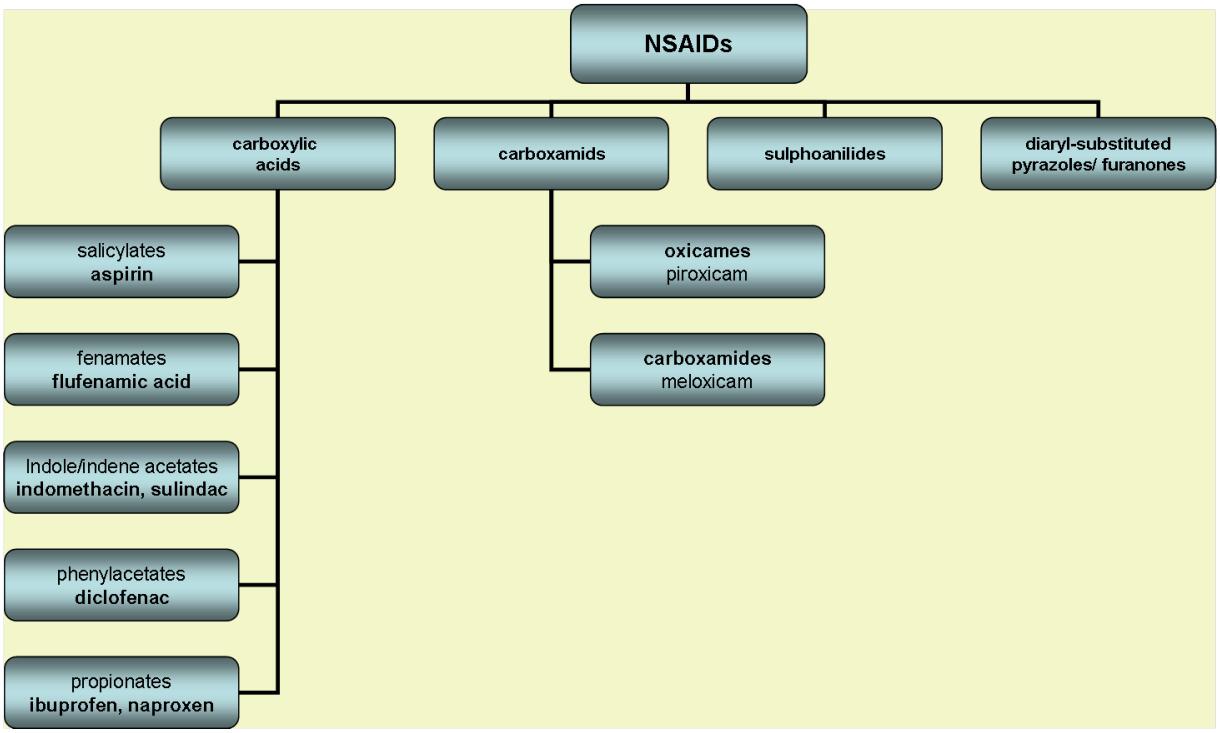


Fig.1: *Classification of NSAIDs due to their structural properties according to Rainsford et al. 2007.*

1.1.2 Chemoprevention by the use of NSAIDs

Since several years, it is known, that a regular intake of NSAIDs accounts for a beneficial prognosis related to a variety of cancer indispositions by interference with the arachidonic acid (AA) pathway. These class of drugs are shown to reduce the risk for certain cancer types as their target, the prostaglandin (PG) producing cyclooxygenases (COX), are upregulated and PG biosynthesis is increased in the tumor microenvironment [4]. As promotion of cancer progression is described in literature by the regulation of tumor-associated angiogenesis, modulation of the immune system, the regulation of cell migration and cell invasion and the inhibition of apoptosis triggered by the COX-2 produced PG, inhibition of both COX-1 and COX-2 was a possible strategy for chemopreventive therapies [5]. An increasing body of evidence linked the AA metabolism pathways and PG biosynthesis, in particularly elevated COX-2 levels, to the promotion and progression of many different cancers, such as lung, breast, colorectal and bladder cancer [6-9]. There is growing experimental and clinical evidence indicating that regular use of aspirin® and NSAIDs reduced the risk of developing cancer, in particularly of colon cancer [10]. Since the 1970s work in several disciplines increasingly has suggested that aspirin® or other NSAIDs may reduce the occurence or progression of colorectal cancers and polyps [11]. In literature,

miscellaneous reports are found about colon cancer and its prevention by the use of NSAIDSs due to COX inhibition [12-14]. Thus, with the current therapy, progressive results are achieved but, like most medications, NSAIDs are not risk-free. Dyspepsia, gastritis, peptic ulcer, gastrointestinal bleeding, hemorrhagic stroke and perforation of gastroduodenal ulcers are thought to be responsible for an imbalance in the maintenance of the intestinal mucosa due to unselective COX inhibition as COX-1 was presumed to be the cause for the undesirable effects [15-18]. In 1991, COX-2, an isoform expressed in inflamed tissues by induction of various factors like cytokines, tumor promotors and inflammatory stimuli, was discovered and hypothesized to be only present under inflammatory and painful conditions [19-21]. Following studies indicated that inducible COX-2 was largely responsible for PG production associated with inflammation in contrast to the housekeeping gene COX-1, whose inhibition appeared to cause more gastrointestinal damage [22, 23]. This provided the rationale for the development of selective COX-2 inhibitors. In a relatively short period several substances entered the market in order to circumvent the mentioned side effects. Rofecoxib (Vioxx®), celexocib (Celebrex ®), valdecoxib (Bextra ®) and parecoxib display a range of agents with chemically different structures of COX-2 selective inhibitors. Etoricoxib (Arcoxia®) failed to enter the US market because of refused approval by the FDA in 2007 [20, 24, 25]. Etoricoxib was approved on the german market in 2004 [26]. Unfortunately, rofecoxib had to be withdrawn from the market due to observed elevated cardiovascular risks [27-29]. Experiments and clinical studies showed decreased vascular prostacyclin production, which is a potent platelet-aggregation inhibitor synthesized by human arteriell and venous tissues from AA [30]. Despite COX-2 inhibitor mediated decreased prostacyclin production there was no concomittant change in thromboxane (TX) levels, a potent platelet-aggregating agent synthesized within the platelets. Inhibition of prostacyclin activity can lead to a predisposition state of thrombosis, hypertension and artherosclerosis. Human studies revealed, that the mentioned cardiovascular events due to decreased prostacyclin levels maybe diminished under coxib therapy [25, 31-35]. In pathophysiological conditions, COX-derived vasoconstrictors such as TXA_2 may act as mediators of myocardial infarction and thrombotic strocke [35]. The role of COX-2 evoked in tumorigenesis in the 1990s. An association between regular consumption of NSAIDs and cancer was made. The reason for the observed effect of reduced incidence of colon cancer was not entirely clear, but it was argued that the protection was exerted via

the COX-2 pathway. Mainly, aspirin® and sulindac were observed to act beneficial on tumor incidence [23, 36]. Further elucidation of the underlying chemopreventive mechanisms by the use of NSAIDs pointed out that not only COX-derived AA metabolites are involved but products formed via the lipoxygenase (LO) pathway named leukotrienes (LTs). Increasing evidence suggested moreover either intervention with the 5-lipoxygenase (5-LO) or LT antagonism. More importantly, a few studies in animal carcinogenesis models showed that inhibition of the 5-LO pathway also may inhibit carcinogenesis.[37]. Investigations made over the past years showed 5-LO inhibitors to act chemopreventively in carcinogenesis of lung, prostate, breast, skin and, extensively studied, in colon cancer and as well in both acute and chronic leukemia [38]. As the AA cascade provides two distinct pathways for intervention, targeting the major enzymes producing metabolites, either PGs or LTs, in a dual manner seems to be a reasonable basic approach as COX and 5-LO pathway appear to act synergistically in the regulation of cell proliferation and neoangiogenesis and all the enzymatically AA-derived metabolites are blocked [39]. Licofelone, former known as ML-3000, is the most prominent candidate in an advanced phase of clinical development, but investigations were currently interrupted [40]. However, molecular mechanisms responsible for chemopreventive properties of NSAIDs, which are still matter of intensive research, are needed to elucidate the effect more detailed. This would contribute to the development of further strategies for inhibitors of involved enzymes lacking typical severe side effects.

1.1.3 Prostaglandin biosynthesis

PGs are biologically active lipid mediators which, together with LT's, prostacyclins and thromboxanes belong to the group of eicosanoids. PGs were discovered early in the 1930's and continuing research led to isolation and characterisation of the biologically active endoperoxide intermediates PGE_2, PGH_2, thromboxane A_2 (TXA_2) and prostacyclin by Bergström, Samuelsson and Vane [32, 41]. All mediators constitute fatty acids with 20 carbon atoms and include a cyclopentane ring, except TXA_2. Substitutions in this ring confer differences in the major PG groups which are classified by letters [42]. PGs are involved in a variety of inflammatory conditions; in particularly in inflammatory conditions of the skin, arthritis, asthma and certain types of cancer [43-45]. They are derived from the precursor AA released from phospholipids

from cellular membranes by enzymes of the phospholipase A_2 superfamily upon activation of various inflammatory stimuli via G-protein coupled receptors [46]. All cells with exception of red blood cells show the capability to synthesize PGs. Previously mentioned, COX enzymes were discovered to exist in two different isosforms. Each isoform catalyzes two reactions and posseses two different activities. The cyclooxygenase activity is used to catalyze the addition of molecular oxygen into the substrate AA resulting in the endperoxide intermediate compound prostaglandin G_2 (PGG_2). Secondly, a peroxidase activity of the COX enzyme catalyzes the reaction of conversion of PGG_2 to PGH_2. PGH_2 is an unstable intermediate for all further steps in the COX pathway which are catalyzed by a number of cell-specific isomerases and lead to the formation of prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$), prostaglandin I_2 (PGI_2 = prostacyclin) and TXA_2 [47, 48].

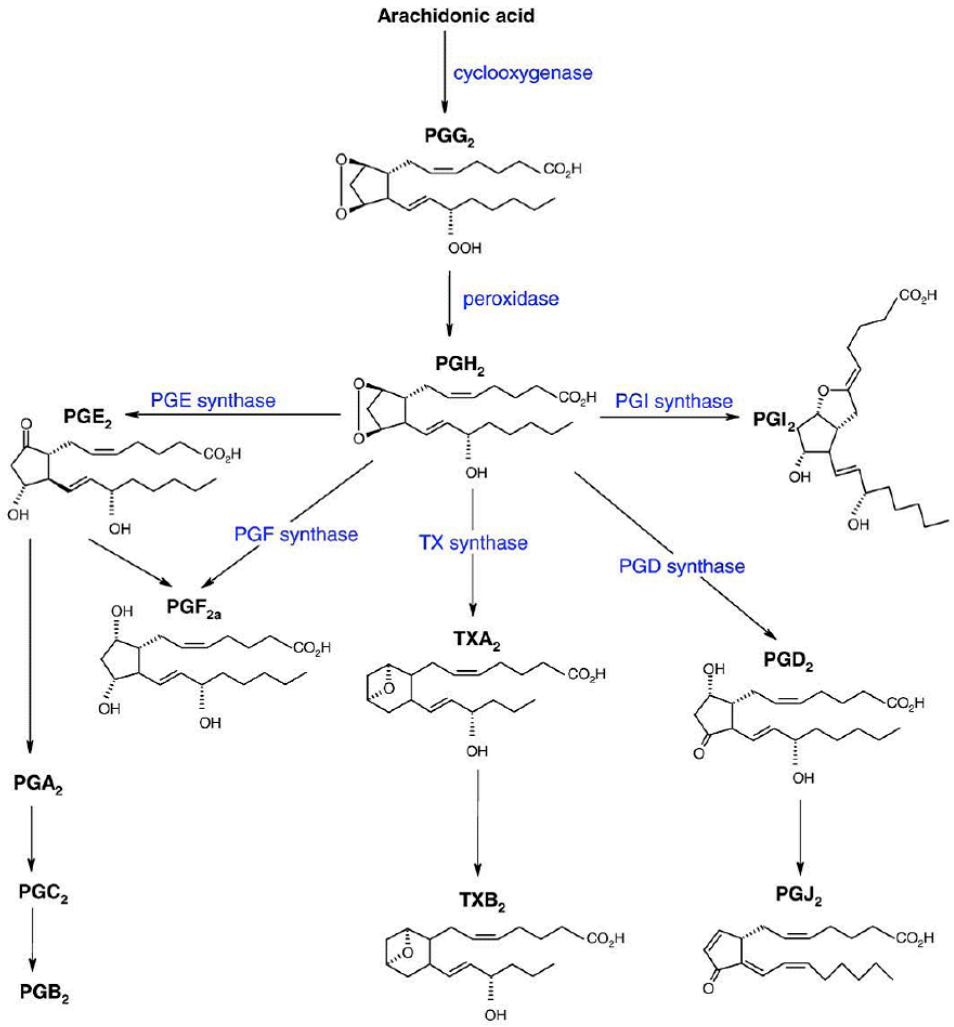


Fig. 2: *COX pathway with main intermediates and products catalyzed by COX and its isomerases including chemical structures. According to Hyde et al. 2009.*

1.1.4 Sulindac - a well established NSAID

The following section provides an overview about the pharmakodynamic and pharmakokinetic properties of the drug sulindac ending with a short description of investgations on gene regulatory functions related to the drug's chemopreventive activity.

Pharmakodynamics

Sulindac is a well-characterized compound and can be classified to the group of NSAIDs. So far, the mode of action for the active metabolite of sulindac, sulindac sulfide (Ssi) comprises inhibition of PG biosynthesis by interfering with both COX enzymes with only a slight preference for COX-1 [49]. In several countries sulindac is approved for the treatment of painful and inflammatory conditions (e.g. USA, spain, france) as it has anti-inflammatory, analgesic and antipyretic properties. Studies evoke participation of sulindac in artherosclerotic processes as experiments in Apo E -/- mice demonstrated reduced neointima formation under sulindac therapy after injury in both hyperlipidemic and normolipidemic mouse model [50]. Furthermore, a role of sulindac in the genesis of Alzheimer's disease is proposed as sulindac may modifiy γ-secretase, a membrane-bound multi protein complex catalyzing the final cut of the Alzheimers's disease related amyloid precursor protein [51]. Among these properties, sulindac is an extensively studied low-ulcerogenic drug with chemopreventive activities. In 1983, observations noting regression of large bowel polyps in patients with Gardner's syndrome (familial colonic adenomas, bone tumors and soft tissues) treated by the NSAID sulindac were made [52]. Additionally, long term effectiveness of sulindac in colorectal adenomas was determined in patients suffering from familial adenomatous polyposis (FAP) and long term use of sulindacseems to effectively reduce dysplasia grade of colorectal adenoma [53]. Further, Ssi was observed to induce cell cycle arrest and apoptosis in mammalian cell lines and targeting of cell cycle check-points is presumed in premalignant cells [54, 55]. Recent work reported that the inhibition of colon tumor cell growth by Ssi is associated with phosphodiesterase-5 (PDE) inhibition in breast cancer [56]. These effects seems to be concomitant with suppression of the ß-catenin signalling in prostate cancer cells [57]. The anti-inflammatory action of NSAIDs is mainly related to inhibition of COX-2 whereas unwanted effects are due largely to their inhibition of COX-1 [58]. Interestingly, the prevention of colorectal polyps in humans by sulindac materializes by inter-

acting with COX-independent targets [59]. However, mechanisms behind the chemopreventive action of this drug still remain unclear and need to be further elucidated.

Pharmakokinetics

Sulindac is an indene acetic analogue of the NSAID indomethacin [60]. As addressed before, the prodrug sulindac is devoid of COX inhibition. After oral administaton, 90% of the drug is absorbed and a two-step biotransformation follows. First, sulindac is oxidized to the metabolite sulindac sulfone, which exerts no COX inhibitory activity, and is then reversibly reduced to the active metabolite sulindac sulfide (Ssi). Ssi has a selective COX-1 inhibitory activity [49]. Plasma half life of sulindac sulfone is about 7 hrs while Ssi has a half life of 18 hrs. Plasma protein binding of Ssi occurs up to 95 % [60]. The usual dose of sulindac is to 200 mg – 400 mg bid (bis intake daily, treatment of rheumatoid arthritis)[59].

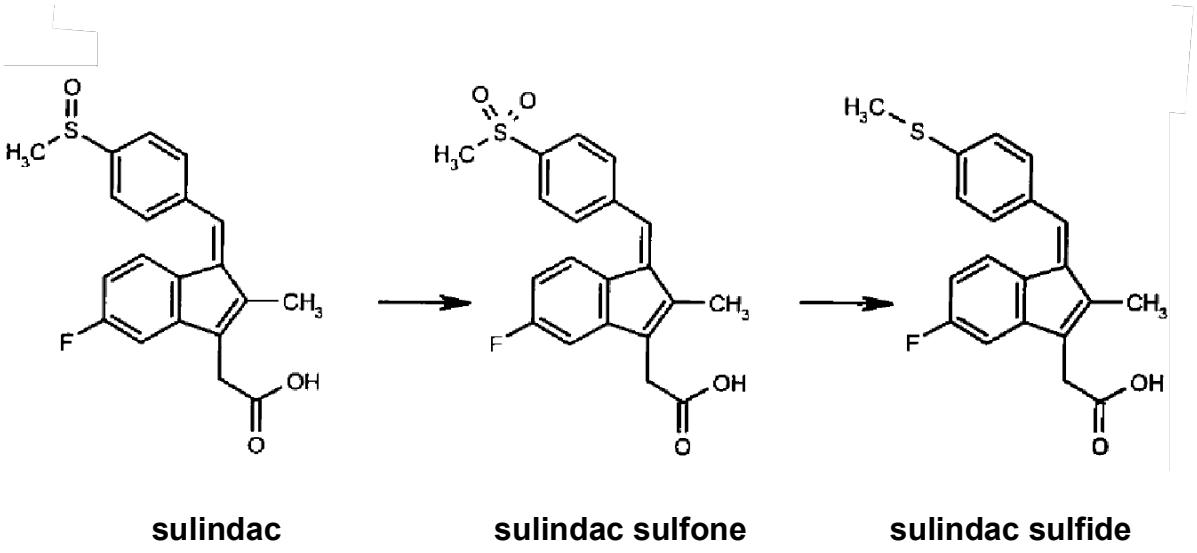


Fig. 3: *Metabolism of sulindac to the active metabolite sulindac sulfide via the sulindac csulfone, an inactive metabolite.*

Regulation of gene expression by Ssi

Lastly, as the chemopreventive effects of NSAIDs seem to be partly COX-independent, it was demonstrated that NSAIDs alter the gene expression patterns which are involved in cancer development, such as NSAID-activated gene-1 (NAG-1), a critical gene regulated by a number of COX-inhibitors and chemopreventive agents [61]. Approaches to elucidate Ssi's chemopreventive molecular mechanisms were undertaken and it was determined that Ssi induces the NAG-1, a gene belong-

ing to the transforming growth factor-beta (TGF-ß) superfamily, resulting in tumor cell growth suppression of ovarian cancer cells [62].

1.1.5 NO-releasing NSAIDs

Nitric oxide-donating NSAIDs (NO-NSAIDs) constitute conventional NSAIDs covalently attached to a NO-releasing moiety, often linked via a spacer molecule. As NSAIDs damage the gastrointestinal mucosa due to inhibition of cytoprotective PGs the strategy of an NSAID locally providing NO was born as nitric oxide (NO) can mimic the PG action. NO-NSAIDs indeed could fulfill protection of the gastric mucosa in contrast to their given parent NSAIDs [63-65]. So far, several molecules with NO-releasing moietys have been synthesized and are currently under investigations in different scopes [66]. NO-donating drugs are promising agents for treatment of cardiovascular diseases, asthma, hypoxic-ischemic brain injury, glaucoma and Alzheimer's disease which is underscored by existing preclinical models and clinical trials [67-71]. The most prominent NO-releasing drug displays NO-aspirin (NO-ASA) which provides the strongest evidence that NO is not responsible for the cytoprotective mechanism of NO-releasing drugs in the stomach as pharmacokinetic studies have shown that NO-ASA traverses the stomach structurally intact [72, 73]. Furthermore, potent esophageal protection was recently shown by NO-ASA which seems to be due to induced enhancement of esophageal microcirculation and inhibition of expression and release of proinflammatory cytokines [74]. Nevertheless, a lot of other compounds were tested. NO-naproxen (naproxcinod) is one candidate, which reached the market so far among the current investigated NO-releasing drugs and an improved safety on the gastrointestinal mucosa was assessed in human studies [75]. Further evaluation led to a beneficial impact of naproxcionod in osteoarthritis of the knee in clinical trials [76]. NO-NSAIDs are an emerging class of compounds with chemopreventive properties in various cancers demonstrated by either pre-clinical models including cell culture models or animal tumor models. NO-ASA, NO-sulindac and NO-ibuprofen were shown to reduce the growth of cultured HT-29 human colon adenocarcinoma cells much more potently than their corresponding NSAIDs providing evidence for anticancer effects of NO-donating drugs [77-80]. Even though the mechanisms behind these effects have been pursued from different perspectives, their complex mode of action possibly involves cell kinetic effects, effects on cell sig-

nalling pathways and on detoxifying enzymes [66]. Mechanistic studies on NO-ASA report a large array of molecular targets such as involvement of mitogen-activated protein kinases (MAPK) signalling, inhibiton of expression and catalytic activity of inducible nitric-oxide synthase (iNOS) and inhibition of the transcriptional factor NF-kB and interference with drug metabolizing enzymes [81-84]. COX-2 effects of NO-NSAIDs are still unclear. However, despite these wide spectra of molecular targets, the defined mechanisms of NO-releasing NSAIDs are still a matter of research.

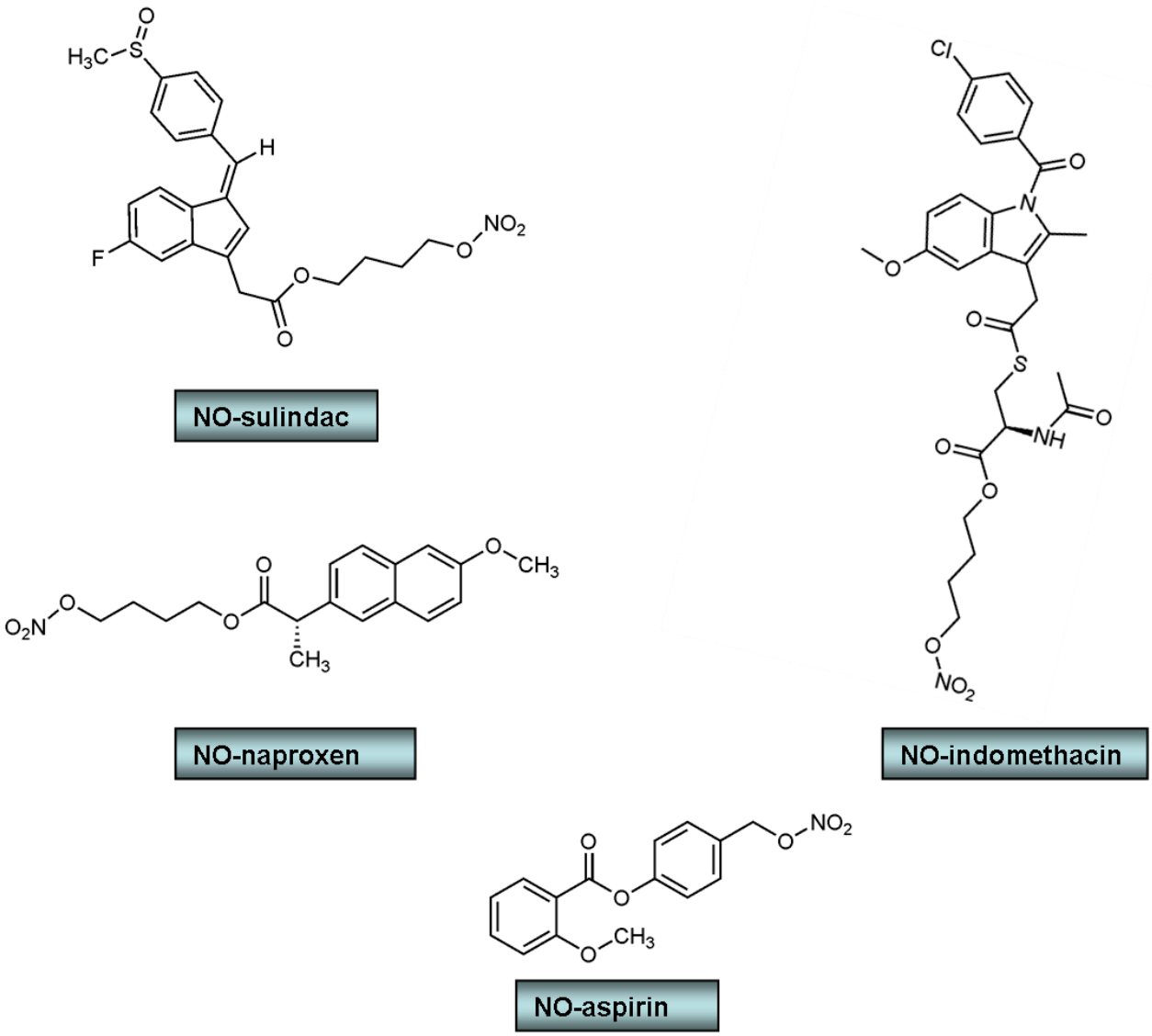


Fig. 4: *Chemical structures of prominent NO-releasing drugs*

1.2 Leukotrienes

In 1976, certain lipid mediators converted from AA by the catalytic activity of the 5-LO enzyme were discovered and named leukotrienes (LTs) [85]. LTs belong to the family of eicosanoids [86]. The following section gives an overview about their biological role in the pathogenesis of different types of diseases, about the restricted cellular LT biosynthesis and about the major enzyme primarily involved in the catalysis of the AA cascade, the 5-LO. Following a detailed description of structure and function of the 5-LO protein, the section ends with a summary of the current existing inhibitor compounds of 5-LO.

1.2.1 Leukotrienes – key players in inflammation and immune reactions

Physiological role of leukotrienes

The word leukotriene comprises „leuko“, from white blood cells and „triene“ meaning three conjugated double bonds. LTs derive from the metabolism of AA, a twenty carbon polyunsaturated fatty acid and constitute a subgroup of potent inflammatory, oxygenated mediators [87] within the field of eicosanoids with several physiological functions in a receptor-mediated fashion [88]. The primary sources of LTs are mature leukocytes but the pattern of lipid mediators produced varies within different cell types. In humans, leukotriene B_4 (LTB_4) is produced in neutrophils, dendritic cells and B-lymphocytes whereas leukotriene C_4 (LTC_4) is mainly found in eosinophils and human mast cells. Monocytes and macrophages are found to form more LTB_4 than LTC_4 [89]. Leukocytes release LTs displaying hormone-analogue properties and show local actions which result in paracrine communication affecting neighboring cells. However, LTs can show autocrine actions as well when they immediately affect the source cell they derive from [89]. Target cells of LTs can be leukocytes, epithelial cells or smooth muscle cells. The first step in LT biosynthesis using AA as subtrate mediated by the enzyme 5-LO, generates the unstable [90] epoxid leukotriene A_4 (LTA_4) as major product [91]. Further transformation by LTA_4 hydrolase produces LTB_4 which can be processed to the cysteinyl-leukotrienes (cys LTs) C_4, D_4 and E_4, also formerly known as slow-reacting substances of anaphylaxis [92]. When inflammation is initiated upon stimulation by stress or infection, LTB_4 known as a potent chemotactic agent, stimulates cell adhesion of leukocytes to the endothelial cell

surface [93] and is responsible for the recruitment of further leukocytes or neutrophils displaying several important functions for the ongoing process of inflammation like additional LTB_4 synthesis by the recruited cells [89]. In consequence, increased phagocytosis of neutrophils and macrophages occurs in addition to neutrophil degranulation [94], liberation of reactive oxygene species and lysosomal enzymes [95] and aggregation [96]. Taken together, LTB_4-driven leukocyte recruitment from the bloodstream into tissues causes a dramatic increase in tissue cellularity, an important factor of inflammation [89]. LTB_4 was also found to activate the peroxisome proliferator-activated receptor α (PPAR α), one of the key players in carbohydrate, lipid and protein metabolism [89, 97]. Beside LTB_4, also the cys-LTs LTC_4, LTD_4 and LTE_4 possess biological activities. Their actions lead to contradiction of airway smooth muscle cells resulting in bronchioconstriction [98]. Further effects promoted by cys-LTs constitute the induction of mucus secretion by bronchial mucosa [99], constriction of both venous and arterial vascular smooth muscle cells, thus representing a regulatory function of cys-LTs in vasoconstriction [100, 101]. The involvement of cys-LTs in allergic reactions is due to the mediated vascular penetration of plasma into tissues while affecting endothelial cells and resulting in edema [102, 103]. Another contribution to inflammation by cys-LTs is the attraction of other blood cells like leukocytes, especially monocytes and eosinophils [89]. All LT effects are mediated by the interaction with specific receptors. The LT receptors can be classified into the group of G-protein coupled receptors (GPCRs) mediating their downstream signaling either via the activation of G_i proteins resulting in decreased formation of cyclic adenosine-monophopshat (cAMP) or via G_q proteins resulting in the activation of phospholipase C (PLC) [89, 104]. These triggers promote downstream signalling by means of kinase activation, involved in cellular and tissue responses [92]. LTB_4 administers its functions by two receptor types, including the high affinity BLT_1 (maximal effect at 1-100 nM) and the low affinity BLT_2 receptor modulated by concentrations of 0.1 - 1.0 µM [92, 105, 106]. For the cys-LTs two G-protein coupled receptors could also be identified which are named cys-LT_1 and cys-LT_2. Differences between cys-LT receptors occur in ligand affinities to LTC_4, LTD_4 and LTE_4 and the type of G protein recruited (G_q) and by the ability of the cys-LT receptors to modulate cellular Ca^{2+}-signalling [89].

Pathophysiological processes associated with enhanced leukotriene formation

It is well known that lipid mediators such as LTs participate in a variety of diseases related to inflammation and allergic processes. It became clear that bronchial asthma is one of the major LT-driven disorders. As mainly cys-LTs were discovered playing a regulatory role in the pathogenesis of asthma enforcing airway hyperreactivity and the frequency of bronchospasms, the focus was set on the development of anti-LT drugs, particularly on cys-LT antagonists [92, 107, 108]. Currently, the cys-LT_1 receptor antagonists montelukast, zafirlukast and pranulukast are approved for the treatment of asthma whereas zileuton was the first direct inhibitor of the 5-LO enzyme which reached the market. Although therapy focuses on cys-LTs, recent studies propose LTB_4 to exert an important regulatory role in models of asthma [109]. Other diseases where LTs are involved are rheumatoid arthritis, allergic rhinitis, psoriasis and inflammatory bowel disease [110, 111]. Ongoing research suggested a role of LTs in the pathogenesis of cardiovascular disorders [92]. The pathogenesis of artherosclerosis, stroke, myocardial infarction and aortic aneurisms is considered to be connected to significantly elevated expression of the participating LT-forming enzymes and increased LT formation was correlated with the mentioned diseases [112-115]. Animal models and even human studies support this hypothesis [116]. Genetic studies on a possible link between the LT / 5-LO pathway and cardiovascular diseases in knockout mice revealed that 5-LO and other enzymes producing LTs are crucial players in cardiovascular diseases. Subsequently, human population genetic studies associated enyzmes involved in LT biosynthesis with cardiovascular disorders. Studies have also identified variants of the 5-LO gene promotor and the five-lipoxygenase-activating protein (FLAP) as risk factors in human artherosclerosis [117-124]. Recent reports provide evidence that excess amounts of LTs could contribute to impaired fracture healing and a 5-LO $^{-/-}$ mouse model showed enhanced and accelerated healing concomitantly by decreased LT levels [125]. Moreover, a role of eicosanoids, especially 5-LO products, in the formation of amyloid beta protein, a major player in Alzheimer's disease was reported [126]. A role of LT's in the pathogenesis of renal disease and an immunoregulatory role in kidney transplantation is currently debated [127]. Another novel indication of anti-LT therapy could be demonstrated with the treatment of osteoporosis as certain 5-LO metabolites seem to

modify bone resorbing osteoclasts, but show disadvantages on the bone providing osteoblasts [110].

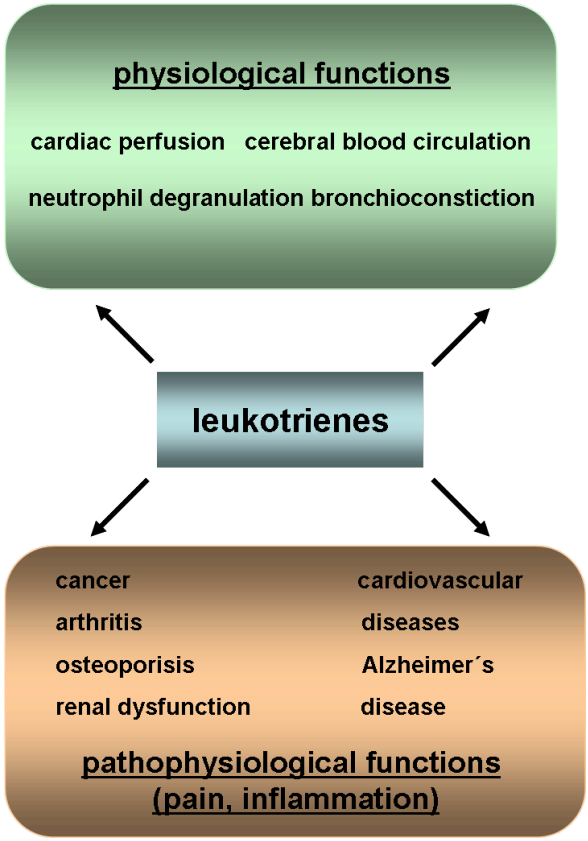


Fig.5: *Summary of physiological and pathophysiological effects of leukotrienes.*

1.2.2 The role of 5-lipoxygenase in cancer and tumorigenesis

An increasing body of evidence suggest a participation producing enzymes in tumorigenesis and the role of 5-LO and its metabolites, mainly LTB_4, are widely discussed to participate in the neoplastic progression in certain types of cancer, as was found in animal models [37, 38, 128, 129]. In 2002, data from lung tumors in mice suggested a role of 5-LO, since the 5-LO inhibitior zileuton and the FLAP inhibitor MK-886 were able to reduce tumor multiplicity in mice [130]. A variety of different malignant tumors are thought to be affected in their development already during the earliest stage of carcinogenesis by 5-LO products, namely prostate, pancreas and breast cancer [110, 131, 132]. The 5-LO metabolite LTB_4 seems to be involved in murine melanoma growth [133]. Also, 5-LO is proposed to play a major role in promoting cell proliferation and therefore tumorigenesis in human brain cancer [134]. Current investigations revealed evidence indicating that 5-LO and LT biosynthesis

are related to malignant diseases not only because of 5-LO overexpression in primary tumor cells and tissues, but also because the addition of 5-LO products to cultured cells led to increased proliferation, cell viability and activation of antiapoptotic signalling pathways [135, 136]. Notably, enzymes related to AA metabolism and linked to 5-LO binding were shown to be overexpressed [137]. Involvement of 5-LO and LTs in the growth and viability of malignant cells were documented first with an antisense approach [138]. Further studies using si-RNA approaches revealed a role for the 5-LO product 5-oxo-(*6E, 8Z, 11Z, 14Z*)-6, 8, 11, 14-eicosatetraenoic acid (5-oxo-ETE) as reduced 5-LO expression significantly impaired prostate cancer cell viability and LT receptor silencing was capable to suppress growth of human colon cancer and neuroblastoma cells [139-141]. Very recently, the 5-LO gene (Alox5) was identified to be crucial in leukemia stem cell proliferation and studies with 5-LO $^{-/-}$ mice underline previous findings by explaining impaired chronic myeloid leukemia (CML) cell proliferation evolved by 5-LO loss [142]. Additionally, the Alox5 gene seems to display a target structure in the specific treatment of tyrosine-kinase-inhibitor-resistant CML as alox5-deficient stem cells show defective ability to develop CML and the approved 5-LO inhibitor zileuton as well as the tyrosine-kinase-inhibitor imatinib led to improved survival of CML-affected mice [143, 144].

1.2.3 Cellular leukotriene biosynthesis

5-LO as the main enzyme responsible for LT formation catalyzes the first two steps in LT biosynthesis and participates in the first enzymatic reaction by its oxygenase activity. It metabolizes AA by abstraction of one hydrogen atom at C-7. After oxygenation at C-5 an intermediate product termed 5-hydroxyperoxyeicosatetraeonic acid (5-HpETE) is formed [145, 146]. As 5-LO possesses another enyzmatic activity, namely the leukotriene A_4 synthase activity, the second metabolic reaction composes the abstraction of a hydrogen bond at C-10 and dehydration lead to the allylic epoxide (conjugated triene C-5,6 epoxide) leukotriene A_4 (LTA_4) [147-149]. 5-HpETE as a catalytic product of 5-LO can alternatively rapidly be reduced to the corresponding alcohol 5-hydroxyeicosatetraenoic acid (5-HETE). Intracellular conditions influence the pattern of the produced metabolites. Cellular glutathione peroxidases (GP-x1) play a decisive role for the reduction of 5-HpETE but also a pseudoperoxidase activity of 5-LO seems to be involved [150]. Subsequently, 5-HETE can be oxidized by 5-

hydroxy-eicosanoid dehydrogenase which is the case in the presence of oxidative stress and reactive oxygen species (ROS) resulting in the synthesis of 5-oxo-ETE. This intermediate displays a precursor structure for several additional eicosanoids via enzymatic linked catalysis or nonenzymatic pathways [151-153]. The unstable LTA_4 intermediate can be enymatically hydrolyzed by LTA_4-hydrolase leading to the dihydroxyacid LTB_4 but this reaction is cell type-specific and depends on the enzymatic equipment of the cell. Furthermore, the conversion of LTA_4 to the LTC_4 is catalyzed by leukotriene C_4 synthase (LTC_4 synthase) or LTC_4 synthase isoenzymes microsomal glutathione S transferase typ 2 (MGST2) and type 3 (MGST3) [110, 146]. Cys-LTs, such as LTD_4 are produced by a subsequent enzymatic elimination of the gamma glutamoyl portion of glutathione of LTC_4. Cleavage of the amino aicd residue glycine in LTD_4 finally yields LTE_4 [92].

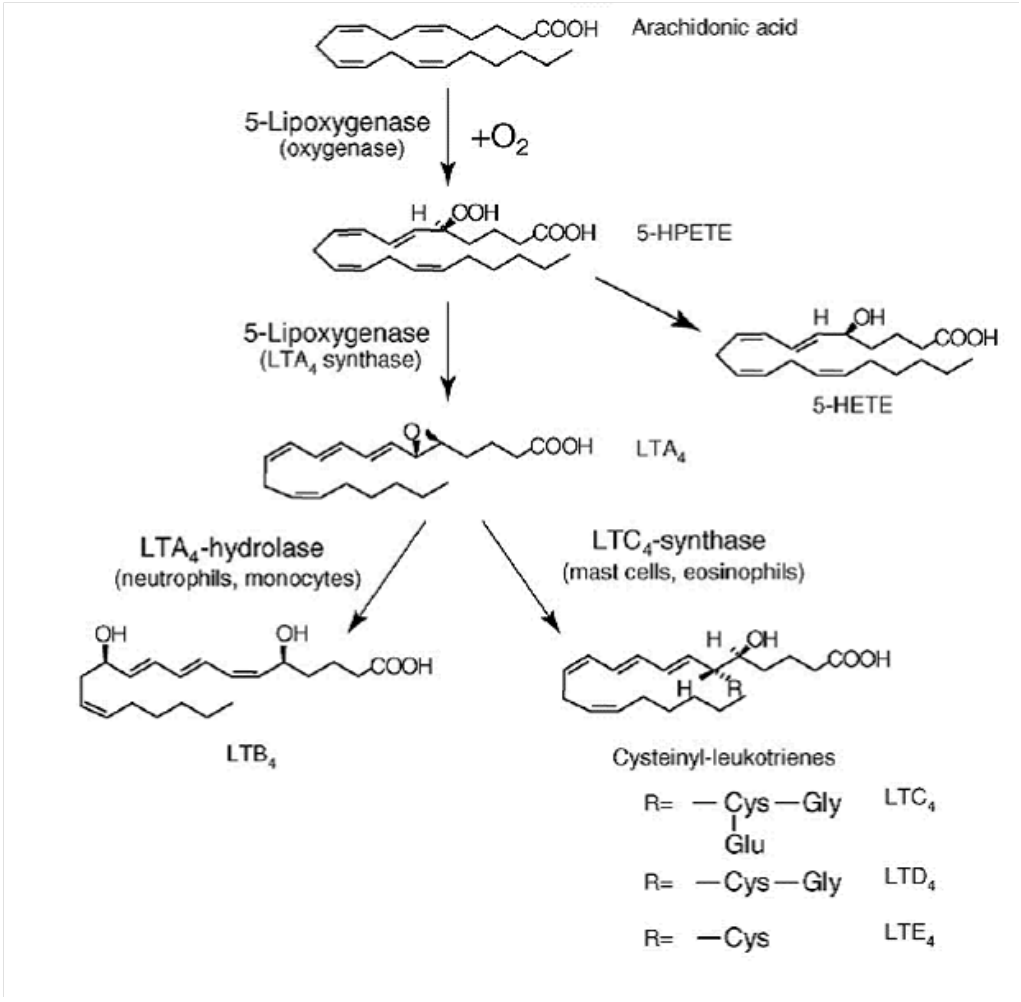


Fig. 6: *Cellular leukotriene biosynthesis based on the conversion of from cell membrane released AA. 5-LO catalyzes in a dioxygenation reaction the formation of 5-HpETE and LTA_4. 5-HpETE is reduced to 5-HETE and LTA_4 is either converted by LTA_4 hydrolase leading to LTB_4 or by LTC_4 synthase to the cys-LTs LTC_4, LTD_4 and LTE_4. Figure according to Radmark et al., 2007.*

1.2.4 The 5-lipoxygenase protein – master enzyme in leukotriene formation

The expression pattern of 5-LO protein varies between different cell types and the capability of 5-LO protein expression was found to be restricted to cells with myeloid origin underlining a pivotal role of 5-LO during cell maturation. 5-LO is mainly expressed in leukocytes which is in line with its discussed functions in immune responses. Also 5-LO is found in granulocytes, macrophages, mast cells, dendritic cells and B-lymphocytes whereas platelets, endothelial cells and T-cells obviously do not express 5-LO [145]. In contrast, a recent publication points out T-cells as a source of 5-LO expressing cells [154]. Further observations revealed an increase in 5-LO mRNA and protein level in differentiated myeloid cells compared to undifferentiated cells using differentiation inducers like dimethylsulfoxide (DMSO), retinoic acid, calcitriol and transforming growth factor ß (TGF-ß) [155]. During cell differentiation in the bone marrow the increase in 5-LO mRNA is thought to reflect the upregulation of 5-LO expression. This is also the case for granulocyte-macrophage colony-stimulating factor (GM-CSF), which is able to augment 5-LO expression in human granulocytes. Further, high levels of 5-LO mRNA occur in peripheral blood leukocytes from asthmatics as well as in macrophage-derived foam cells in the human vessel wall, which has been linked to the involvement of LT biosynthesis in cardiovascular diseases [112, 156]. It has additionally been reported that in lung macrophages accelerated levels of 5-LO protein were examined during the immigration of blood monocytes into tissues affiliated with their differentiation into macrophages [157, 158].

1.2.5 Related lipoxygenases

LOs form a familiy of lipid peroxidizing enzymes found in animals as well as in plants. So far, 18 different mammalian LO sequences have been published [159]. Apart from 5-LO, two other subfamilies of mammalian LOs exist: 12- and 15-LO, which exert functional lipid mediators with distinct profiles of action [159, 160]. The complex array of formed metabolites gained by these LOs are tissue- and species- specific and can result in pro- and anti-inflammatory effects [161]. One lipid mediator derived from the AA cascade by catalytic activity of 12-Lipoxygenase (12-LO) via stereospecific oxygen insertion to AA is 12-(S)-hydroperoxyeicosatetraenoic acid (12-HpETE) which is rapidly reduced to 12-(S)-hydroxyeicosatetraenoicacid (12-HETE) seemingly func-

tioning as a stop signal for inflammatory processes [162].12-LO isoformes are discovered in platelets (platelet-12-LO), in leukyocytes and in the epidermis and are often termed after the cells they were first identified in [163]. 12-LO is widely discussed as a potential pathogenic enzyme which is thought to be involved in a great number of diseases, namely diabetes, cardiovascular dysfunctions such as atherogenesis and angiogenesis, hypertension, renal disorders, sickness of the central nervous systems, circumstantial Alzheimer's and Parkinson's disease [164]. Other recent publications claim a prominent role of 12-LO as a potential new therapeutic target in the regulation of epithelial ovarian cancer cells since decreased cell growth and survival was observed after silencing 12-LO gene [160]. Additionally, a possibility of 12-LO serving as a unique marker and target in prostate cancer stem cells raised lately [165]. 15-hydroxyeicosatetraenoic acid (15-HETE) represents another lipid signalling molecule derived from the subgroup of LOs which is assumed to exhibit opposed properties than the 12-LO metabolite 12-HETE [161]. Antiatherogenic characteristics by inhibition of oxidative stress are attributed to 15-LO and studies in rabbits revealed that overexpression of 15-LO protein showed protective mechanisms against artherosclerosis. One isoform, 15-LO-1 was thought to play a role in the oxidative modification of low-density lipoproteins and foam cell formation and a proatherogenic role in mice models was established [166]. Further, 15-LO-1 seemed to display a key component in the cell proliferation process in epithelial cancer cells, in particular prostate, colorectal and breast cancer [167]. In 2008, another pathophysiological role for 15-LO-1 was proposed argueing for an involvement of 15-LO-1 in allergic airway inflammation such as asthma. These findings were supported by 15-LO-1 deficient mice models and underline a putative therapeutic implication of 15-LO-1 inhibitors in the treatment in airway inflammation [168]. In the same year, anti-carcinogenic effects, reflected in enhanced apoptosis and a decreased proliferation rate were reported and a role for 15-LO-1 in survival of colorectal cancer cells was demonstrated [169]. As 12- and 15-LO enzymes are expressed in a variety of tissues in the human body and their metabolites contribute to certain diseases, the development of isoform-specific inhibitors will be of great interest as these enzymes represent promising drug targets [164]. 15-LO-2, mainly expressed in normal human prostate epithelial cancer cells, additionally, has a physiologic tumor suppressor function [170].

1.2.6 The 5-lipoxygenase gene and promoter region

Discovered in 1989, the 5-LO gene consists of 14 exons divided by 13 introns with a total length of 79 kilobases (kb). Exons 4 - 8 comprise five conserved histidin residues and six more conserved acidic and basic residues followed by the latter region. This region is supposed to be involved in the iron binding and a short sequence displaying homology to the interface binding domain of lipases is located in this region. Therefore, it was concluded that AA binding occurs in this region and it is encoded in exon 8 and part of exon 9 [171]. The putative promotor region was characterized in 1990 and is located 5.9 kb upstream from the ATG transcriptional initiation site. It is lacking the typical TATA and CCAAT boxes near the transcription initiation site and thus shows properties of a housekeeping gene [171-173]. Sp1, a wide spread transcriptional activating factor, is thought to be able to bind to a sequence of five from eight existing repeated GC-boxes designated by Hoshiko and co-workers as the transcription factor binding region. The transcriptional factor Egr1 is also recognized at this binding site [173, 174]. These GC-rich sequences were discovered as essential regions in the 5-LO promotor for expression of native 5-LO in Mono-Mac-6 (MM6) cells and also a novel Sp1 binding site 20 base pairs upstream from the transcriptional initiation site was detected [175]. Sp1 is an ubiquous zinc-finger containing protein shown to crosstalk with numerous other proteins. High expression levels of Sp1 are correlated with cells undergoing differentiation and high amounts of Sp1 seem to be needed for the induction of tissue-specific genes [176]. Sp1 is modified by a variety of posttranscriptional events such as sumoylation, acetylation, phosphorylation or glycosylation for example, resulting in augmented target promotor binding and transcriptional activity [177, 178]. Basal regulation of 5-LO transcription is influenced by Sp1 via binding to the GC-4 boxes [179]. Beside Sp1 and Egr1, other consensus sites for promoter regulating proteins are characterized, namely Sp3, Egr2, GATA or NF-kB, but only Sp1, Sp3 and Egr1 show functional effects on the 5-LO promoter [180]. Further upstream in the promoter region, in intron 4 and M, calcitriol-responsive elements were recognized [181]. Very recently, 5-LO mRNA isoforms were detected in human leukocytes lacking catalytic activity and therefore possibly representing a novel regulation mechanism of the 5-LO pathway [182].

1.2.7 The 5-lipoxygenase protein – structure and function

The next section deals with the three-dimensional structure of the 5-LO enzyme and its properties. So far, for structural elucidation efforts where made on modeling the 5-LO structure on the basis of the rabbit reticulozyte 15-LO crystal structure and these studies emerged that a two-domain structure appears to be present, that is a catalytically active domain and a small regulatory C2-like domain (C2ld). This C2ld is responsible for binding several factors and adjusting biological functions of 5-LO, similar to the C2 domain of $cPLA_{2\alpha}$ [148, 183]. Very recently, the group of Newcomer *et al.* reported the crystal structure of human 5-LO where a newly discovered 5-LO destabilizing sequence was mutated [184]. The human enzyme is a monomeric 78 kDa protein [185]. As the enzyme can be divided into two structural different domains, these will be handled separately in the following.

The catalytic domain of 5-lipoxygenase

For studies on the catalytic domain of 5-LO the published structure of rabbit 15-LO was used as a template due to existence of high similarity between the two catalytic domains of the two proteins [183]. The C-terminal catalytic domain (amino acid residues 121-673) of 5-LO contains a non-heme-iron atom which cycles between the ferric and the ferrous state during enzyme catalysis and is chelated by three histidine residues (His-367, His-372, His-550), an asparagine (Asn-554) and the C-terminal isoleucine carboxy group (Ile-673) [85]. The sixth ligand of the coordination sphere can be proposed as water. The iron functions as the prosthetic group of the enzyme and stays in the ferrous state (Fe^{2+}) while the enzyme is resting in the cytosol. Mononuclear non-heme Fe^{2+}- containing enzymes present this feature typically, called the 2-His-1-carboxylaed facial triad [186]. The aforementioned peroxidase activity of 5-LO is able to reduce lipid hydroperoxides generating Fe^{3+} in the active site. An originated AA radical at C-7 can then be attacked by molecular oxygen resulting in a 5-hydroperoxy radical intermediate. The resulting Fe^{2+} can now donate an electron to the radical and form the 5-hydroperoxy anion from which 5-HpETE is released after regenerating the active Fe^{3+} [148]. Fatty acid hydroperoxides are required to activate enzyme activity in a cell-free system turning the non-heme-coordinated iron into the ferric state. 5-, 12- and 15-HpETE as well as 13-hydroperoxylinoleic acid were all shown to be similar in their capacity to stimulate 5-LO in crude leukocyte homogenates. In contrast, the corresponding HETEs were totally inactive as stimulators of 5-

LO, indicating that an intact hydroperoxy function is necessary for enzyme activation. Other standard hydroperoxides, e.g. hydrogene peroxide or *t*-butyl hydroperoxide were inactive, as they do not contain a long chain fatty acid part [187]. The performance of the enzyme, once switched to the active form, is characterized by a linear propagation phase with maximal conversion rates followed by an irreversible turnover dependent inactivation (suicide inactivation) [188]. The catalytic domain further features three phosphorylation sites, at Ser-271 for mitogen-activated protein kinase activated protein kinase-2 (MAPKAP-2), at Ser-663 for extracellular regulated kinase-2 (ERK-2) and at ser-523 for PKA. Phosphorylation or dephosphorylatione events regulate 5-LO enzyme activity [189-191]. Additionaly the specific phosphorylation at Ser-271 serves to inhibit nuclear export of 5-LO which is consistent with the nuclear import action induced by phosphorylation of Ser-532 [192]. Using photoaffinity labelling, a general nucleotide binding site was discovered and utilized for affinity chromatography in terms of protein purification as ATP appears to interact specifically at this sequence [193, 194]. In the same way, the binding site of the natural substrate of 5-LO enzyme, AA, was proposed [195]. The carboxyterminal domain predominantly contains α-helices [196].

The regulatory C2-like domain of 5-lipoxygenase

C2-domains generally are known as Ca^{2+} binding proteins and display a conserved structural motif forming an eight-stranded antiparallel ß-sandwhich mediating binding to a variety of ligands including divalent cations, phospholipids and other proteins [197, 198]. Sequences involved in the core ß-sandwhich are highly conserved between C2-domains whereas the sequences of the loops are not [199]. C2-domains have been identified in membrane-interacting proteins, e.g. $cPLA_2$, synaptotagmin and protein kinase C (PKC) [200]. The N-terminal regulatory domain of human 5-LO is composed of two four-stranded antiparallel beta-sheets (ß-sandwhich) and can be categorized into the PLAT (polycystin-1, lipoxygenase, α-toxin) family which do have structural features similar to C2-domains [196, 201]. In a model of the 5-LO structure based on the crystal structure of the ferrous form of rabbit reticulocyte 15-LO, amino acid residues 1-121 are assigned to the small regulatory domain [202]. Within the emerged homology model of 5-LO the regulatory domain is termed “C2-like-domain” in comparison to other proteins as differences exist in the constitution of the ß-sheets [203]. For human 5-LO, several functionalities are ascribed to locations within the C2ld suggesting the small domain as a possible target for enzyme inhibition.

Divalent cation binding

C2-domains are often refered to as Ca^{2+}-dependent lipid binding domains but are not obligatory Ca^{2+} binding as they display a family of versatile protein modules with diverse functions. Ca^{2+} ususally binds to negatively charged amino acid residues such as aspartate (asp) side chains and glutamine (glu) which can serve as ligands for two or three Ca^{2+} ions and are conserved as well [199]. First, the C2ld of 5-LO possesses the ability to associate two Ca^{2+} ions shown in Ca^{2+}-overlay studies, electrophoretic mobility shift assay, gel filtration and equilibrium dialysis. Mutagenesis studies of the loops between the ß-strands (ligand binding loop 2) revealed decreased enzyme activity due to reduced Ca^{2+}-binding suggesting that these residues are important for Ca^{2+}-stimulated 5-LO enzyme activity [200]. Ca^{2+} was found to bind 5-LO reversibly with a kd of ~ 6 µM and the stochiometry of maximum binding averaged around two Ca^{2+} ions per 5-LO molecule [203]. In cell-free assays half-maximal activation of enzyme activity was reached with 1 - 2 µM Ca^{2+} and full activity is reached with 4 -10 µM Ca^{2+}. Ca^{2+} mediates the interaction with phospholipds and membrane fractions [204, 205]. As binding of Ca^{2+} occurs at the lipid-water interface and increases hydrophobicity of the protein, the concept is currently proposed that Ca^{2+} stimulates 5-LO activity due to promoting membrane association. This is, as aforementioned, mediated by the C2ld of the enzyme [200]. Interestingly, Ca^{2+} is not required for 5-LO activity if high concentrations of phosphatidylcholine (PC) vesicles, displaying natural constituents of cellular membranes, arise. Then, 5-LO can be distributed in AA vesicles and product formation proceeds in the absence of Ca^{2+} [206]. Furthermore, the interaction of Ca^{2+}, located at the C2ld, renders the enzyme resistant to GP-x1 possibly by increasing the affinity towards lipidhydroperoxides needed for enzyme activation [207]. Mg^{2+} ions, even though with lower affinity compared to Ca^{2+}, are able to bind and activate 5-LO activity in a dose-dependent manner (0.1 – 1 mM) in the presence of PC vesicles [208].

Membrane targeting

C2 domains are Ca^{2+}-dependent membrane targeting modules found in many cellular proteins involved in signal transduction or membrane trafficking [209]. Upon cellular stimulation, 5-LO is activated by Ca^{2+}-mediated binding to nuclear membranes. It is suggested that the N-terminal ß-barrel domain of 5-LO functions to facilitate mem-

brane binding which is a prerequisite for 5-LO activation [210, 211]. It is believed that the attachement of 5-LO to the nuclear membrane is influenced by non-specific interactions with factors like membrane surface charge or the distinct lipid composition of the nuclear membrane. Several phospholipids, such as phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylglycerol (PG), diacylglycerol (DAG) and PC have been examined, as in vitro 5-LO activity is rather low except in the presence of membrane fractions or phospholipids. Only PC was found to increase 5-LO activity, even if 5-LO association has been shown also for PE and PS. A higher affinity for zwitterionic PC than for anionic PS or acidic lipids was detected leading to the conclusion that this might be due to 5-LO binding to PC-rich nuclear membranes [210]. Also, the membrane surface charge seems to play an important role in the modulation of 5-LO activity. This was inferred from a trend of suppression of 5-LO activity with an increase in negative surface charge of membranes [212, 213]. Another important determinant of membrane binding strength of 5-LO, enzyme activity and penetration into the hydrophobic core is membrane fluidity. An activity and binding strength increase could be correlated to the dregree of lipid acyl chain cis-unsaturation and peaked with 1-palmitoyl-2-arachidonolyl-*sn*-glycero-3-phosphocholine (PAPC). A group of trp-residues was found to penetrate into the hydrocarbon region of PAPC membranes and a model of 5-LO bound to phospholipid membrane was proposed based on the structure of rabbit reticulocyte 15-LO [214]. Therein, trp-13, trp-75 and trp-102 are likely to interact with the membrane and it is suggested that trp-75 penetrates deeper into the membrane's surface than other residues. Trp-13 inserts in the hydrophobic part of the membrane and trp-102 is positioned close to the lipid-water interface. Kulkarni *et al.* proposed trp-102 as the most important trp residues for the 5-LO C2ld and membrane interaction which does not show conflicts as trp residues located at the polar headgroup/hydrocarbon borderline of membranes are able to facilitate protein interactions via H-bonding and nonpolar interactions [215]. The three above mentioned trp residues are also involved in the 5-LO stimulation induced by 1-oleoyl-2-acetyl-sn-glycerol (OAG). OAG was detected to directly bind to the putative PC-binding pocket. Similar to Ca^{2+}, OAG antagonizes the effect of GPx-1 on 5-LO activity [216].

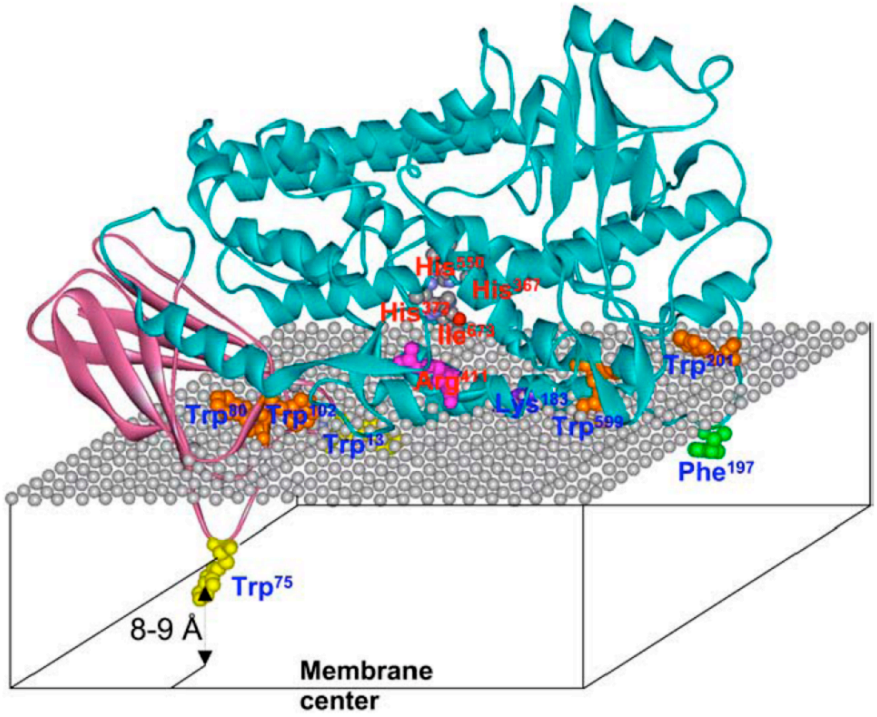


Fig. 7 *Model of 5-LO bound to a phospholipid membrane according to Pande et.al, 2005.*

Protein-protein interaction

C2 domains have been identified to bind a variety of different ligands including other proteins beside Ca^{2+} and phospholipids [198]. The human coactosin-like protein (CLP), a small 16kDa (142 amino acids) cytosolic protein, affiliated into the group of F-actin-binding proteins and similar to coactosin found in *Dictyostelium discoideum*, was discovered to attach to 5-LO and upregulate enzyme activity. This is mediated via an interaction with the three trp residues (13, 75,102) located in the regulatory domain of 5-LO comprising the PC binding pocket [217, 218]. Ca^{2+}-induced 5-LO activation occurs in the absence of PC indicating a membrane mimicking scaffold function of CLP. In presence of PC up to 3-fold increase of LTA_4 amounts was evident. These facts point to an intervention with the three trp residues in the 5-LO ß-sandwhich as all effects were abrogated with a 5-LO protein mutant lacking trp-12, trp-75 and trp-102 (5-LO 3W-mutant) [218]. Single trp mutants showed 50 % activity in presence of low PC and AA on wildtype 5-LO [219]. Thus, all three trp residues seem to contribute equally to PC binding to support 5-LO enzyme activity [220].

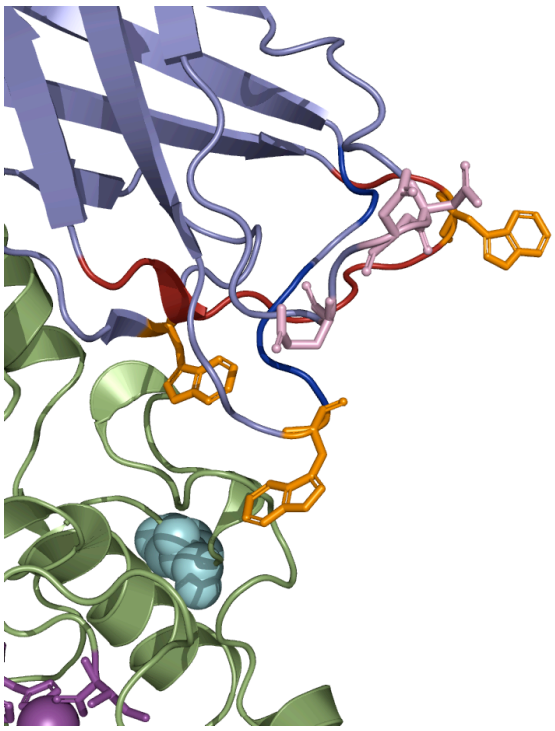


Fig. 8 *Zoom into the model of human 5-LO at membrane interaction site*

Model of the 5-LO C2-like domain in purple and the catalytic residue in green.The three trp-residues are marked in orange indicating the positions which are responsible for membrane interactions and are mutated in case of the 5-LO-3W mutant protein lacking membrane binding capability.Model by B.Hoffmann.

Recently, an interaction of 5-LO with a large protein, consisting of 1912 amino acid was discovered which belongs to ribonclease III family involved in the biogenesis of micro RNAs and is named dicer. Human dicer has been shown to interact with cellular proteins via its N-terminal domain but it was found that dicer-5-LO interaction is provided via its C-terminus and the lipid binding site within the C2ld domain of 5-LO as the association of both proteins is interrupted when trp residues are mutated to ala. This association may provide a novel link between micro-RNA mediated regulation of gene expression and inflammation [221]. It is also suggested that 5-LO and FLAP expression may be involved in the cerebellar LT formation and/or could possibly interfere with the dicer-mediated micro-RNA formation and processes of neuroplasticity [222]. Apart from these findings, interactions with transforming growth factor type ß-receptor-I-associated-protein 1 (TRAP-1) were reported by screening a human lung cDNA library and may be involved in the TGF-ß-induced upregulation of 5-LO expression and activity observed in HL-60- and MM6-cells [217].

3D model of human 5-lipoxygenase

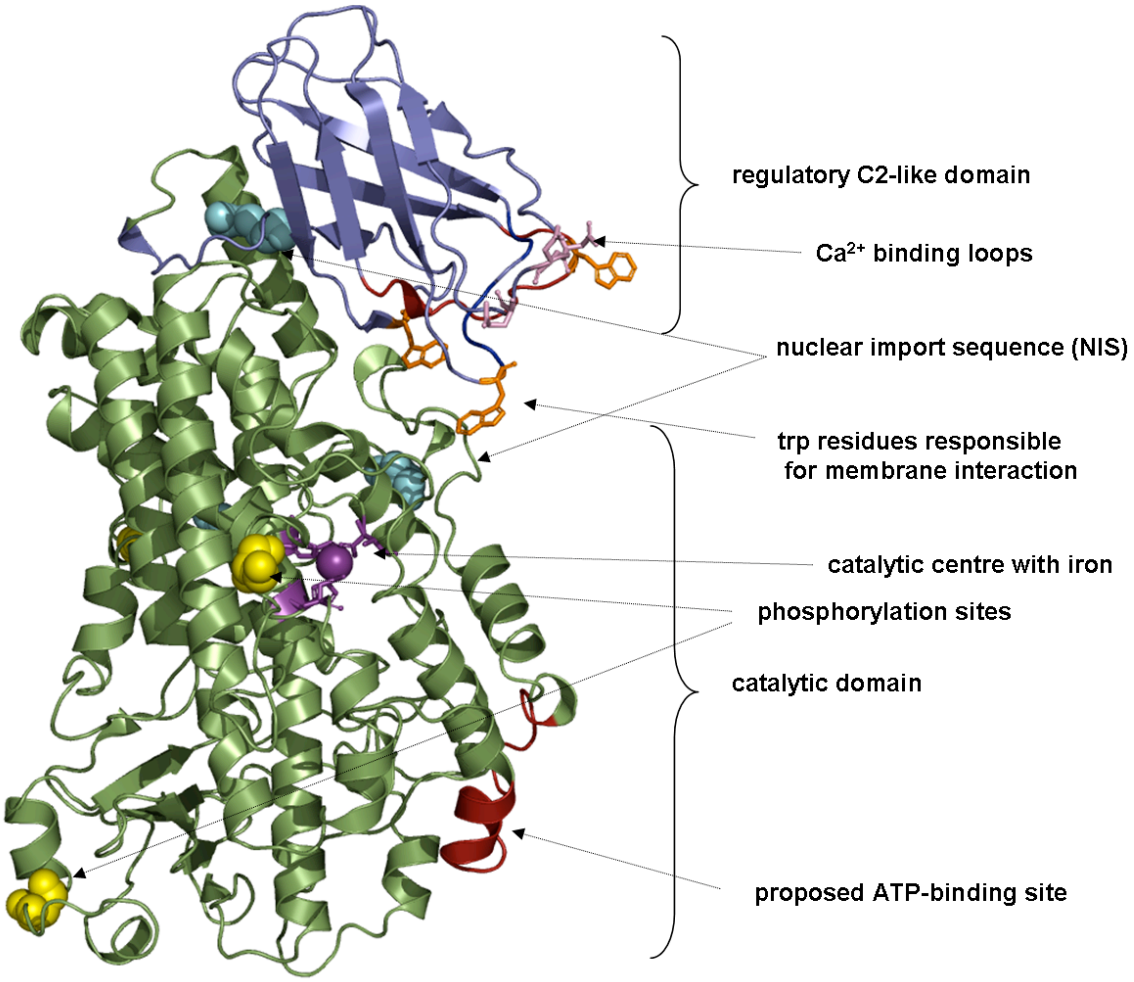


Fig. 9. *3D model of the human 5-LO with structural functionalities (calculated by B.Hoffmann)*

1.2.8 Cellular regulation of 5-lipoxygenase activity

As 5-LO is the key enzyme in LT biosynthesis and lipid mediators play a critical role in inflammatory processes, 5-LO expression and enzyme activity are tightly controlled on cellular levels.

Cellular environment

Given that 5-LO is a non-heme iron containing enzyme which needs initially oxidation to the ferric form, protein activity is regulated by the cellular redox tone. The pres-

ence of sufficient lipidhydroperoxides is therefore pivotal. A cellular enzyme involved in redox events is the selenium containing Gpx-1, which reduces 5-HpETE and thereby governs the actual acitivity of leukocyte 5-LO via regulation of the endogenous tone of hydroperoxides [223]. The addition of Gpx-1 to purified 5-LO prevents enzyme inactivation and in presence of reactive Gpx-1 5-LO product formation is suppressed by reducing the required hydroperoxides. Adversely, the inhibition of Gpx leads to enhanced LT levels and upregulation of 5-LO activity. Subsequently, both Gpx-1 and phospholipid-hydroperoxideglutathioneperoxidase (Gpx-4) do affect cellular 5-LO activity cell-type dependently [224-226]. The B-lymphocytic cell line BL41-E95A and also immature HL-60-cells display less resistance to Gpx-4 emerging almost no 5-LO activity unless diamide or H_2O_2 was added in contrast to 5-LO prepared from granulocytes [227, 228]. In monocytes and the monocytic cell line MM6 inhibition of 5-LO is caused by Gpx-1 [229]. To avoid a great loss of 5-LO activity, Gpx is also used for protein stabilization under storage conditions [225].

Stimulatory factors

One of the key determinants stimulating 5-LO activity are Ca^{2+}-ions. Early studies revealed a role of Ca^{2+} in LT biosynthesis as calcium-ionophore (A23187) initiates SRS-A production [230]. Interestingly, the concentrations needed for stimulation of recombinant 5-LO reaching from 1 - 2 µM (half-maximal activation) to 4 - 10 µM (maximal activation) are considerably higher than the estimated intracellular Ca^{2+} concentrations required for 5-LO product formation in leukocytes indicating that cellular events and context adjust the Ca^{2+} requirement [145, 231]. In polymorphonuclear leukocytes (PMNL) natural agents like N-formyl-leucyl-phenylalanine (fMLP) or platelet-activation-factor (PAF) induced cellular Ca^{2+} influx and effectively activate cellular LT formation when. An increased release in AA and synthesis of LTB_4 was detectable [232]. However, recent studies indicated reduced Ca^{2+} requirements in the presence of DAG after cell stimulation with A23187 or thapsigargin. These findings might predict that Ca^{2+} alone without DAGs is not able to activate 5-LO catalysis [233]. Furthermore, OAG is able to stimulate 5-LO activity in a magnitude comparable A23187 in the presence of exogenously added AA [234].

Phosphorylation of 5-lipoxygenase

The addition of a phosphate group to a protein is a very specific regulatory mechanism and can either result in upregulation of a certain protein function or inhibition and the family of kinases play an important role in signal transduction. 5-LO was observed to be phosphorylated at three different serine residues. First, phosphorylation of 5-LO was caused by a member of the protein kinase A family, the catalytic subunit of protein kinase A (PKA). A correlation of phosphorylation by PKA and 5-LO enzyme activity was observed in alveolar and peritoneal macrophages [191, 235]. Mutagenesis studies showed that Ser-523 is the key residue for phosphorylation of 5-LO inhibiting the catalytic unit of the enzyme and reducing LT generation in LT producing cells (neutrophils, basophils, mast cells, eosinophils and macrophages) [191, 236-238]. PKA is usually activated by an increase of cAMP following ligand binding or PGE_2 binding. Polyunsaturated fatty acids (PUFAs) prevent cAMP-mediated downregulaton of 5-LO product synthesis in activated neutrophils [239]. Further, a phosphorylation-driven change in subcellular localization was described leading to accumulation of 5-LO protein in the cytoplasm as Ser-523 is part of a nuclear localization sequence (NLS) [240]. Other kinase pathways impact on cellular LT formation, namely the family of mitogen-activated-protein-kinases (MAP-kinases). Within this family, MK-2 and ERK were found to phosphorylate 5-LO and intervene in 5-LO catalyis. P38 mitogen-activated protein kinase (p38 MAPK) is activated by cellular stress like sodium arsenite (chemical stress) in B-cells and human PMNL and in turn phosphorylates and activates MAPKAP kinase 2 and 3 (MK2/3). MK-2 then phosphorylates 5-LO on Ser-271. 5-LO can be upregulated by the addition of PUFAs suggesting a putative fatty-acid binding site [188, 241-245]. Interestingly, SA and osmotic stress were effective also after chelation of Ca^{2+} in the medium and inside the cells [246]. ERK 1/2 phosphorylates 5-LO at ser-663 and ERK activation was found to mediate cellular activation of 5-LO in the presence of unsaturated fatty acid (UFAs) [190]. In MM6-cells, conditions leading to higher activity were connected with increased 5-LO kinase activity and upregulated 5-LO translocation to the nuclear membrane [242]. The activity of kinases on 5-LO seems to be responsible not only, as mentioned previously, for 5-LO activity and subcellular localization but also for Ca^{2+}-induced membrane association and/or regulation of interaction with other cellular components [85, 247, 248].

5-lipoxygenase: interaction with membranes

Membrane constituents are important activation factors of 5-LO activity and are essential for 5-LO product synthesis as conversion of endogenously provided AA occurs at the nuclear membrane [145]. In unstimulated cells 5-LO is either located in the cytosol (neutrophils, eosinophils or peritoneal macrophages) or in a nuclear soluble compartment associated with chromatin as in alveolar macrophages, Langerhans cells or basophilic leukemia cells [146]. Upon stimulation, cytosolic 5-LO translocates to the nuclear membrane in the cell [85]. Different patterns have been observed for 5-LO localization in various cell types, particularly for peripheral blood leukocytes as compared to alveolar macrophages [249]. Commonly, A23187 is a prominent stimulus for membrane binding of 5-LO in intact cells and induces 5-LO to produce LTs after binding to nuclear membranes [250]. Reversibility in membrane binding could be demonstrated using different stimuli of 5-LO, particularly when cells are stimulated in a weaker fashion with fMLP, IgE-antigen, zymosan or even with A23187 [251, 252]. It is debatable whether 5-LO first translocates and migrates into the nucleus and afterwards binds to the perinuclear membrane from the inside or if 5-LO is attached from the outside of the nuclear membrane. It has been shown that nuclear import of 5-LO takes places independently of enzyme activity and LT formation, underlined by findings of elicitation of rat peritoneal neutrophils by glycogen and after adhesion of human PMN. It was pointed out that both alveolar macrophages and elicited PMNs show increased capability for 5-LO product formaton [203] but with a relatively high Ca-ionophore activation threshold [253, 254]. The exception are eosinophils, where nuclear localization suppresses 5-LO activity. Additionally, LT formation can take place in the absence of nuclear membranes when exogenous AA is supplemented as natural substrate in the incubations [255]. 5-LO import into the nucleus is assessed by the nuclear localization sequence (NLS) which displays a basic amino acid rich sequence and is present in the N-terminal domain as well as close to the C-terminus of 5-LO which could be determined with GFP-5-LO fusion protein in transfected cells [256-258]. It was concluded that several parts of the protein contribute to the nuclear import sequentially, but the ß-barrel domain of 5-LO was found out to be essential for membrane association [259]. Also, nuclear export sequences have been identified in 5-LO and nuclear export was shown when alveolar macrophages are removed from the alveolar milieu [254, 260]. It is still speculated if events leading to

unmasking of the NLS by phosphorylation are relevant in subcellular localization processes of 5-LO [240, 248, 261, 262].

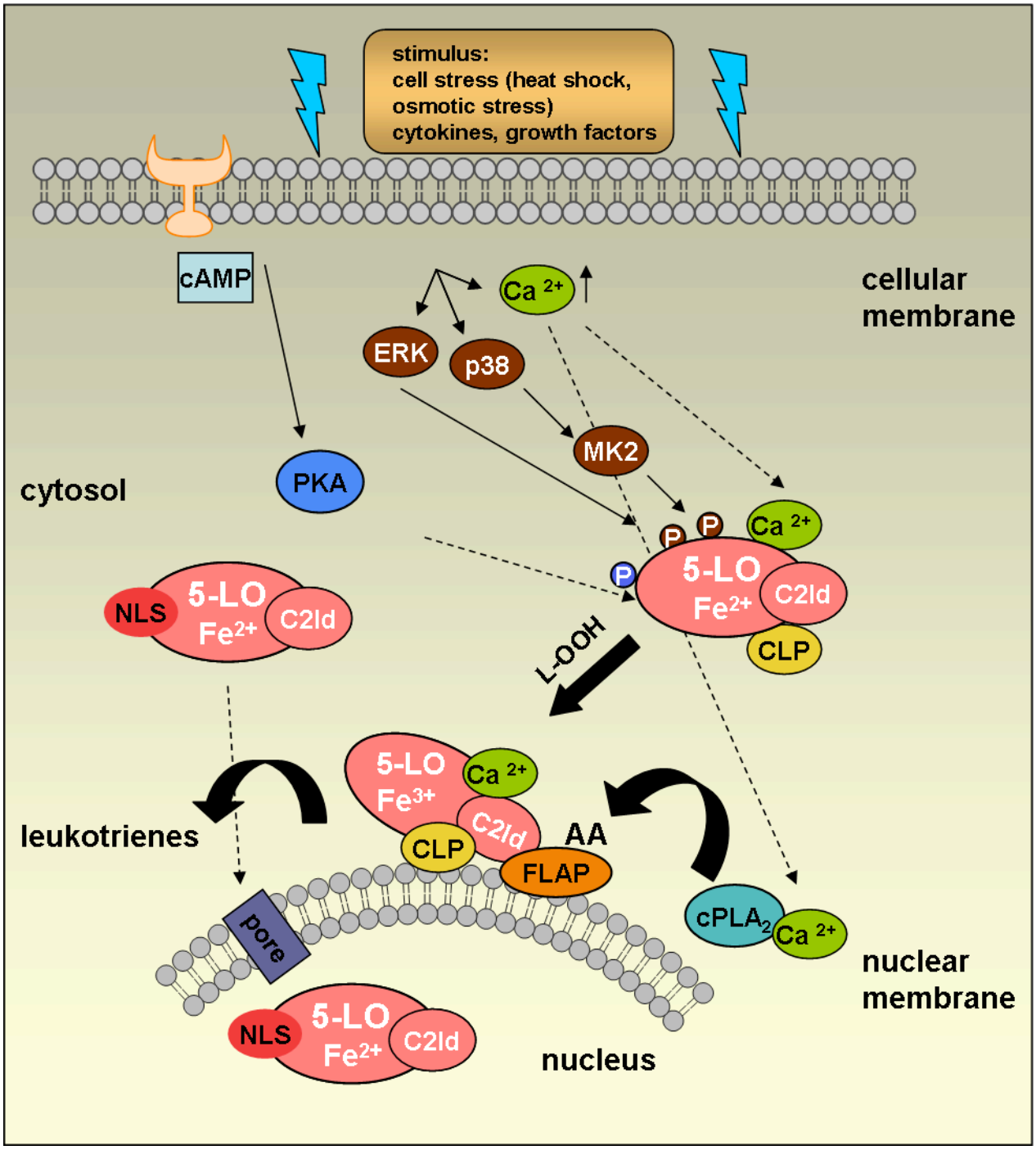
5-lipoxygenase interacting proteins in leukotriene biosynthesis

Finally, interaction of 5-LO with other proteins is an important criterium influencing 5-LO's enzymatic activity in a cellular environment. CLP, as aforementioned, is colocalized with 5-LO at the nuclear membrane after stimulation of human neutrophils with Ca-ionophore and the same distribution pattern for CLP and 5-LO was observed in stimulated MM6-cells [217]. CLP was shown to replace cellular membranes as a scaffold for 5-LO but still Ca^{2+} was required for LT biosynthesis. This led to the hypothesis that the two proteins form a complex which enables cytosolic formation of 5-HpETE respectively 5-HETE when triggered by the addition of Ca^{2+} and exogenous AA. Thus, the mode of binding of 5-LO to CLP might depend on the presence of Ca^{2+}, resembling the "productive" or "non-productive" modes of membrane binding of the protein. It was concluded that CLP promotes LTA_4 formation catalyzed by 5-LO when PC is present. Aside from these facts, CLP regulates the ratio of formed 5-LO metabolites, namely 5-HpETE and 5-HETE resulting in a shift in favour to 5-HETE [218]. Lastly, FLAP assists 5-LO at the nuclear membrane catalyzing 5-LO metabolite origination. FLAP is a member of the membrane associated proteins in eicosanoid and glutathione metabolism (MAPEG) and sequential identities of FLAP and LTC_4 synthase (also MAPEG member) are 31 % [263, 264]. The three-dimensional structure of human FLAP in complex with MK-591 was recently determined by X-ray chrystallography [265]. Another compound, MK-886, was found to block membrane association of 5-LO in human leukocytes and by radiolabelled MK-886, a 18kDa protein was designated and then purified in neutrophils [266, 267]. FLAP is a membrane bound-protein and protein expression of both FLAP and 5-LO are considered to be necessary for Ca-ionophore induced 5-LO catalytic action in transfected human osteosarcoma cells in the absence of exogenously added substrate [268]. As MK-886 did not affect 5-LO membrane association in 5-LO transfected osteosarcoma cells lacking FLAP, an accessory role of FLAP as a membrane anchor was assumed [269, 270]. This is underlined by the suggestion that FLAP may function as a AA transfer protein facilitating access to the natural substrate of 5-LO and it became evident that FLAP is crucial for conversion of endogenous AA [85]. At this point 5-LO inhibitors

are thought to compete with AA or other fatty acids regarding binding to FLAP [271, 272]. Recently, another substrate such as 12-(S)-HETE was discovered [273]. Binding of FLAP to AA was shown, however, no enzymatic activity of FLAP has been reported yet [274].

Gender-specific aspects

Recently, gender-related differences in LT metabolism and 5-LO catalytic activity have been observed on cellular levels (neutrophils) and in the whole blood system. Studies indicate that 5-LO and LTs are androgen regulated. Androgens cause nuclear localization of 5-LO in an ERK-dependent fashion resulting in decreased 5-LO product formation induced by the pathophysiological stimulus fMLP. LT levels detected were lower in males compared to females [275]. These findings may explain recognized gender differences in LT-related diseases which were found to be more prominent in females [276].



Schematic of cellular LT biosynthesis

Fig. 10: *Schematic of the cellular biosynthesis of leukotrienes by 5-LO according to Radmark et al., 2007.*

1.2.9 5-lipoxygenase inhibitors

As LTs play a prominent role in a great variety of diseases including respiratory dysfunctions, allergic reactions, osteoporosis, cardiovascular diseases and cancer, (see section 1.2), great efforts have been made in the development of anti-LT therapy and intervention with 5-LO is a challenge in the development of therapeutics. Despite a role for LTs in various pathologies, the progress made in the development of 5-LO inhibitors is rather moderate. However, novel pharmacological strategies for intervention are of great importance to the field and concepts of inhibition of LT synthesis seem to be promising. The following subsection deals with the general pharmacological concepts in 5-LO inhibition by currently available compounds and summarizes the different molecular approaches to inhibit 5-LO by different intervention strategies [276].

Redox-type inhibitors

Redox-type inhibitors of 5-LO intervene with the enzyme by uncoupling it from its catalytic cycle due to reducing the active site iron in the ferric state keeping it in the ferrous state (Fe^{2+}) and by acting as non-selective antioxidants. Mostly, compounds display lipophilic properties and are derived from plant-origin such as nordihydroguaraic acid, caffeic acid, flavonoids, coumarins and other polyphenols. The first synthetic compounds inhibiting 5-LO were redox-active 5-LO inhibitors such as AA861, L-656,224, phenidone or BW755C [277]. AA861 showed IC_{50} values of 3 µM for LTB_4 and 0.1 µM for LTC_4. BW755C inhibited the generation of LTC_4 with an IC_{50} value of 100 µM indicating that AA861 is about 1.000 times more potent than BW755C. Aditionally, AA861 did not affect COX or 12-LO. Essentially no antagonistic activity to LTD_4 or histamine was observed [278, 279]. Despite their great efficiency in vitro, all agents lacked suitable oral bioavailability and inhibitor profiles possess poor 5-LO selectivity and show adverse side effects as other biological redox systems are influenced by the drugs such as methemoglobin formation and further, reactive radical species can be formed [280]. Due to these properties the compounds were not convincing and failed to enter the market [281].

Iron-chelating compounds

The development of compounds that are able to chelate the active site iron comprise derivatives of hydroxamic-acids and N-hydroxyurea. A very prominent member is the compound BWA4C (hydroxamic acid) which potently inhibits 5-LO in intact granulocytes with an IC_{50} value of 4 nM [282]. BWA4C is a feasible control agent in in vitro assays. Further investigations led to the hydrolytic-stable N-hydroxyurea derivative zileuton, a potent and orally bioavailable 5-LO inhibitor (IC_{50} value 0.5 - 1 µM in stimulated leukocytes) [283, 284]. It leads to therapeutic effects in acute and chronic improvement of airway functions and therefore to a reduced need of first-line therapy with glucocorticoids or ß-agonists. Zileuton finally entered the market with approvement by the FDA in the United States. It is the first available 5-LO inhibitor for the treatment of bronchial asthma, trade name Zyflo® [285]. Though, the drug only showed low therapeutic effects in allergic rhinitis, rheumatoid arthritis and inflammatory bowel disease [286]. Surprisingly, with the exception of zileuton as the only marketed iron-ligand inhibitor of 5-LO, no notable efforts concerning the approval of drugs have been made in the past five years. One approach accomplished a COX-inhibitory moiety, preferentially a NSAID, and a covalently linked iron-chelating moiety leading to the dual COX-5-LO inhibition concept. Recently, zileuton was identified as inhibitor of PG biosynthesis by interfernce with AA release in macrophages [287].

Non-redox type inhibitors

Mostly, a lack of selectivity and no appropriate bioavailability are prominent disadvantages of 5-LO inhibitors that initiated the development of the class of non-redox type 5-LO inhibitors. The designation „non-redox type“ inhibitor implicates only that the compounds themselves do not show redox activity [288]. Non-redox-type inhibitors are thought to compete with the natural substrate of 5-LO, AA or lipidhydroperoxides for binding to 5-LO via the subtrate binding-cleft in the active site. This fact is still matter of research. Some studies suggest an additional allosteric mode of action [111, 289]. The group comprises structurally diverse molecules. Lead structure optimisation led to the orally active potent 5-LO inhibitors ZD-2138 and ZM-230487 (IC_{50} values 20 - 50 nM, respectively) tested in human leukocytes and human whole blood [290]. Elevated peroxide levels and/ or phosphorylation of 5-LO by MK-2 strongly decreased 5-LO inhibitory potency of these compounds in stimulated PMNL and in con-

sequence led to diminished 5-LO inhibition in inflammatory tissues exhibiting a higher peroxide level. Furthermore, non-redox type inhibitors require Gpx for efficient inhibition of 5-LO [289]. A promising candidate, the urea derivative RBx7796, was discovered by Ranbay *et al*. as a novel type of 5-LO inhibitor. The compound showed 5-LO inhibition (IC_{50} values 3.8 µM (cell-free assay) and 5 µM (cell-based assay)) with a characterized competitive, selective mode of action, orally active, stable concerning metabolic features and a lack of cytochrome P450 inhibition [291, 292]. Ongoing research led to L-739.010 which possesses the structure of substituted coumarins to minimize toxicity and was introduced by the Merck Frost group. Simultaneously, related fluorophenyl-substituted coumarins where the thioaryl moiety carrying the hexafluorcarbinol was replaced by an amino-oxadiazol moiety and therfore displaying potent 5-LO inhibition in cell-free assays and human whole blood without acting toxic were claimed [276]. Another structural different set of compounds, a potent imidazole series, represented by CJ-13.610, was developed by Pfizer. CJ-13.610 is a potent 5-LO inhibitor in cellular systems (IC_{50} = 70 nM, neutrophils) and human whole blood (IC_{50} = 230 nM) and its efficacy was not influenced by phosphorylation of 5-LO [293, 294]. Virtual screening studies identified a novel series of tricyclic thiazole-based derivatives where one compound exerts strong 5-LO inhibition in cell-free assays as well as in neutrophils (IC_{50} values 0.15 µM respectively, 0.17 µM) but failed to show inhibitory potency *in vivo* or in human whole blood. The molecular mechanisms of these compounds are unknown or only partly characterized but structure-based evaluation underlines that iron-chelating or reducing properties can be excluded [276].

FLAP-inhibitors

In the late 1980s, MK-886 was discovered, an indole-class compound showing LT biosynthesis inhibition both in vitro and in vivo without interfering directly with 5-LO [295]. At the same time, BAY X1005, displaying similar properties, was developed at Bayer [296]. FLAP was then identified using a photoaffinity analogue of MK-886 with which the photoaffinity-labeled protein could be immunoprecipitated from human leukocyte membranes [266, 268]. Further studies identified binding sites for quinoline- and indole-based inhibitors like MK-591, also possessing FLAP inhibitory properties. Shown by mutagenesis studies, MK-591’s binding site seems to overlap with the AA

binding pocket [297]. With these prominent FLAP-inhibiting compounds, phase I and II clinical trials were undertaken in the treatment of asthma. Despite good clinical safety profiles lacking hepatotoxicity and potent activity against early and late phase lung volume decrease after allergene challenge, none of the compounds were further developed [298-301]. As the crystal structure of inhibitor-bound FLAP was determined by X-ray investigations, details of the binding of FLAP to the nuclear membrane could be clarified. FLAP forms homotrimers within the nuclear membrane and each monomer is composed of four transmembrane helices that are connected by two cytosolic loops and a lumenal loop [302]. Three MK-591 molecules are bound in between monomers in three groves which are arranged on the membrane-embedded surface of the trimer leading to a binding stoichiometry of 1:1 [267].

Dual COX-/ 5-LO or COX-/ FLAP inhibitors

Within the past years, several approaches were made to develop dual inhibiting substances either affecting related LOs (12-LO, 15-LO) or COX enzymes. This is in line with the finding that all these enzymes do have in common that they catalyze reactions with the same substrate, AA. Therefore they might bind to a more or less conserved AA-binding pocket in the active site of the 5-LO enzyme. Compounds thought to interfere with related LOs may have off-target effects that are relevant in terms of adverse side effects [276]. A focus lies on the development of potent anti-inflammatory drugs not showing the well-established gastrointestinal toxicity [303]. A very prominent representative was licofelone (ML-3000) which was developed until clinical trial phase III in 2007 and showed inhibitory potency on both enzymes, COX and FLAP [111, 304]. Unfortunately, the clinical trial phase III for ML-3000 was stopped due to unpublished reasons.

Other 5-LO inhibitors

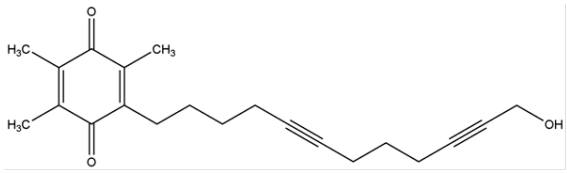
Besides this classification of so far developed and characterized 5-LO inhibitors comprising a wide array of different chemical structures, there are many compounds derived from plant origin known to inhibit 5-LO enzyme activity and therefore to suppress LT biosynthesis. As aforementioned, many phytochemical compounds were tested on their ability to inhibit 5-LO product formation, exemplified by myrtucommu-

lone from the plant *Myrtus communis*, boswellic acids originated from *Boswellia serrata*, namely 11-keto-ß-boswellic acid (KBA) and its acetylic derivative 3-O-acetyl-11-keto-boswellic acid (AKBA) and as well hyperforin, the major ingredient of St. Johns Wort (*Hypericum perforatum*) [241, 305-308]. All these compounds were identified as potent 5-LO inhibitors with corresponding IC_{50} values ranging from 0.1 – 5 µM tested in intact cells and cell-free assays. Myrtucommulone and hyperforin were thereby verified as dual COX/5-LO inhibitors [111]. Hyperforin's mode of action is well characterized and a unique molecular mechanism postulating that it interacts with the C2ld of the 5-LO enzyme [309]. Recently, inhibition of microsomal PGE_2 synthase by hyperforin was described [310].

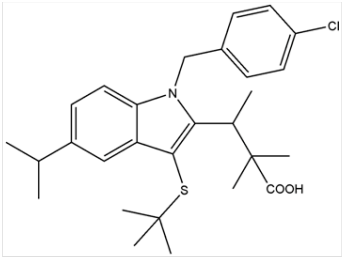
Summary of the current standings in 5-LO inhibitor development

The previous chapter gives a complex picture of LT bioynthesis suppression by 5-LO inhibitors. Despite great efforts which were made in the past und current investigations, only one 5-LO inhibitor (zileuton) has so far reached the market. More potent inhibitors are needed that do not present the typical side effects and that should have excellent pharmacokinetic and pharmacodynamic profiles without toxicity.

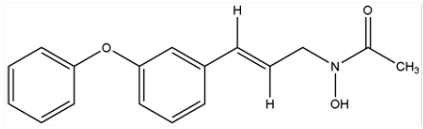
Representatives including chemical structures and potencies for each type of 5-LO inhibitor



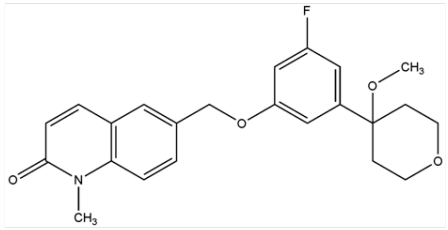
AA-861



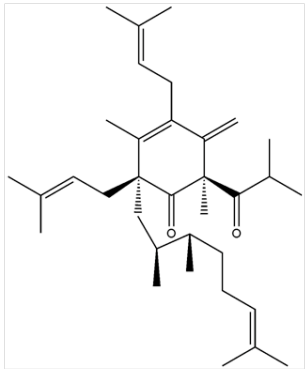
MK-886



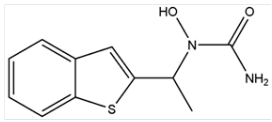
BWA4C



ZD-2138



hyperforin



zileuton

name:	type of inhibitor	IC_{50} (intact cells)	IC_{50} (cell-free assay)
AA-861	redox-active inhibitor	0.1 - 3 µM	0.1 - 3 µM
BWA4C	iron-ligand inhibitor	0.5 - 1 µM	0.1 µM
ZD-2138	non-redox type inhibitor	20 - 50 nM	50 - 100 nM
MK-886	FLAP-inhibitor	2.5 nM	-
Hyperforin	C2-like domain inhibitor	0.09 µM	1.2 µM
Zileuton	iron-ligand inhibitor	0.3 µM	0.3 µM

Fig.11: *Summary of different types of 5-LO inhibitors including their structures and potencies in cellular and cell-free assays*

2 AIM OF THE PRESENT STUDY

LTs constitue a group of lipid mediators derived from the precursor AA in a two-step reaction catalyzed by the 5-LO enzyme. These pro-inflammatory mediators play a pivotal role in inflammatory processes and host defense [311]. Beside inflammatory diseases like asthma, rheumathoid arhtritis, allergic disorders or atherosclerosis, 5-LO has been shown to be involved in tumorigenesis and cancer [19]. Other enzymes involved in the AA cascade such as COX are upregulated and PG production is increased in tumor tissues [4, 312]. As some NSAIDs show beneficial cardiovascular profiles, higher gastrointestinal tolerance and even chemopreventive properties like cell cylce arrest and growth inhibition, intense reseach to evaluate possible targets of these drugs has been performed over the last years.

First evidence for a potential role of NSAIDs in the progression and risk reduction of cancer was derived from clinical observations in studies of patients suffering from FAP and Gardners syndrome who were treated with the well-established NSAID sulindac [52, 313]. An increasing body of evidence indicates that there may exist other mechanisms of NSAIDs than inhibition of prostaglandin synthesis which are responsible for chemoprevention and higher compatibility in humans [5].

Ssi, is a well-established NSAID on the market since the 1970s. The drug possesses strong anti-inflammatory, analgesic and anti-pyretic effects showing a low ulcerogenic and cardiovascular profile [59]. Its neoplastic efficacy has been shown by interactions with COX-independent targets preventing precancerous colorectal polyps in humans [59].Thus, having in mind the exceptional pharmacological profile of Ssi and the pathophysiological effect of LTs, the present studies were undertaken in order to determine and characterize a possible influence of Ssi on LT biosynthesis and furthermore to identify the underlying mechanisms. Moreover, the studies aimed to evaluate a possible regulatory effect of Ssi on 5-LO gene expression. The use of NO-NSAIDs seems to be a promising strategy to inhibit PG biosynthesis. Due to their beneficial chemopreventive and gastroprotective effects, it was additionally attempted to investigate a possible interaction of NO-NSAIDs and 5-LO as a second, potent target.

3 MATERIALS AND METHODS

3.1 Cells and cell culture

3.1.1 Cell lines

Mono-Mac-6-cells

The human cell line MM6 was derived from the peripheral blood of a 64-year-old man suffering from acute monocytic leukemia. MM6-cells show a constitutive expression pattern of mature monocytes regarding phenotypical and functional tasks [314]. 5-LO protein expression and subsequent enzymatic activity can be triggered by treatment of the cells with human TGF-ß (1ng/ml) and 1α,25-dihydroxyvitamin D3 (calcitriol) (50 nM) [315].

HeLa-cells

HeLa cells were cervix carcinoma cells. Due to their 5-LO deficiency, HeLa cells are a suitable assay system for elucidating effects which are induced by transfection of 5-LO plasmids.

3.1.2 Cell culture

MM6-cells were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10 % heat-inactivated (2 hrs at 56°C) fetal-bovine-serum, 100 µg/mL streptomycin, 100 U/ml penicillin, 1 mM oxaloacetic acid, 1x MEM non-essential-amino acids and 10 µg/mL human insulin. The human insulin was kindly provided by Sanofi-Aventis, Frankfurt. HeLa cells were cultured at 37°C in a 5 % CO_2 atmosphere whereas MM6-cells were maintained at 37°C with a 6 % CO_2 atmosphere.

3.2 Isolation of PMNL from buffy coats

Human polymorphonuclear leukocytes (PMNL) were freshly isolated from leukocyte concentrates obtained from the Krankenhaus Höchst (Frankfurt, Germany) or from the Blood Center, University Hospital (Tübingen, Germany). Venous blood was collected from fasted, adult, healthy volunteers, with consent. The subjects had no apparent inflammatory conditions and had not taken anti-inflammatory drugs for at least 10 days prior to blood collection. The blood was centrifuged at 4.000xg for 20 min at

room temperature for preparation of leukocyte concentrates. PMNL were immediately isolated by dextrane sedimentation, centrifugation on Nycoprep cushions (PAA Laboratories, Linz, Austria), and hypotonic lysis of erythrocytes [246]. PMNL ($5x10^6$ cells/ml; purity > 96−97 %) were finally resuspended in phosphate-buffered saline pH 7.4 (PBS) plus 1 mg/ml glucose and 1 mM $CaCl_2$ (PGC buffer).

3.3 Isolation of platelets from buffy coats

After the densitiy centrifugation step during the isolation of PMNL, plasma containing the platelets was centrifuged for 15 min at room temperature at 4.800xg. Pellets were resuspended in PBS/NaCl 0.9 % (v/v) and centrifuged again at 4.800xg for 10 min at room temperature. Cells were finally diluted into PBS buffer pH 5.9.

3.4 Preparation of microsomal fractions from PMNL

$1x10^7$ freshly isolated PMNL were subjected to centrifugation at 4.000xg/20 min and pellets were resuspended in 1 ml homogenization buffer (PBS, sucrose 0.25 M, Complete Mini Protease Inhibitor cocktail tablette (Roche Diagnostics, Mannheim, Germany)). After sonication for 3x20 s and centrifugatioin at 10.000xg/10 min where unbroken cells, nuclei and mitochondria are pellete the resulting supernatants containing soluble enzymes and fragmented ER were again centrifuged at 170.000x g/70 min at 4°C. Microsomal fractions were then kept in 100 µl of homogenization buffer and total protein concentration was determined by using the Bradford method [316].

3.5 Purification of human recombinant 5-lipoxygenase from *E.coli*

The proteins 5-LO-wt and 5-LO-3W were expressed in *E. coli* Bl21 (DE3) cells, transformed with pT3–5LO, and purification of 5-LO was performed by affinity chromatography. *E. coli* were harvested and lysed in 50 mM triethanolamine/HCl, pH 8.0, 5 mM EDTA, soybean trypsin inhibitor (60 µg/ml), 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM DTT and lysozyme (100 mg/ml), homogenized by sonication (3x15 s) and centrifuged at 10.000xg for 15 min followed by centrifugation at 100,000xg for 70 min at 4°C. The supernatant was then applied to an ATP-agarose column (Sigma A2767, Deisenhofen, Germany), and the column was eluted as described previously.

Partially purified 5-LO was immediately used for *in-vitro* activity assays. For some studies 5-LO was further purified by ion-exchange chomatography on an ÄKTA-purifier 100 system (GE Healthcare).

3.6 Determination of protein phosphorylation

PMNL ($1x10^7$/100 µl PGC buffer) were preincubated with the indicated concentrations of Ssi, Sso, sul or vehicle for 10 min. Then, 100 nM fMLP was added, the reaction was stopped after 1.5 min at 37 °C by addition of 100 µl of SDS-PAGE sample loading buffer (20 mM Tris/HCl, pH 8, 2 mM EDTA, 5 % SDS (w/v), 10 % ß-mercaptoethanol), and heated for 6 min at 95°C. Cell lysates were separated by SDS polyacrylamid gel electrophoresis (SDS-PAGE) on a 10 % gel, electroblotted on Hybond ECL nitrocellulose membranes (GE Healthcare, Munich, Germany) and analysed for phosphorylated ERK1/2 and phosphorylated p38-MAPK using an Ettan-DIGE imaging system (GE Healthcare) as described [275].

3.7 5-lipoxygenase translocation assay

Freshly isolated PMNL ($3x10^7$) in 1 ml PGC buffer were pre-incubated with the compounds or vehicle (DMSO) for 15 min at 37°C. Then, 2.5 µM A23187 was added, the samples were further incubated for 5 min, and subsequently chilled on ice to stop the reaction. Nuclear and non-nuclear fractions were obtained after cell lysis by 0.1 % NP-40. Samples were then prepared for SDS-PAGE and Western blotting.

3.8 Determination of 5-lipoxygenase product formation

5-LO products were either determined in cellular systems such as PMNL, platelets and eosinophils or activity of 5-LO was measured by the metabolic rate of 5-LO to its natural substrate arachidonic acid with purified, recombinant enzyme.

3.8.1 Cellular 5-lipoxygenase product formation

PMNL ($7x10^6$) immediately isolated from leukocyte concentrates and resuspended in 1 ml PGC buffer were incubated with the test compounds or vehicle (DMSO) at 37°C

for 10 min. For the competition assay, cells were first incubated with the FLAP inhibitor MK-886 (5 µM) for 10 min and then another 10 min for test compounds. For stimulation with fMLP, cells were primed with 1 µg/ml LPS/0.2 U/ml Ada 30 min prior to the incubation with 1 µM fMLP for 5 min. Test compounds or vehicle (DMSO) were added 10 min before treatment with fMLP. 5-LO product formation was started by the addition of the stimuli that is either 2.5 µM A23187, 10 µM sodium arsenide (SA) or 300 mM sodium chloride (SC) together with or without AA as indicated. The reaction was stopped after 10 min by addition of 1 ml ice-cold methanol and the formed 5-LO metabolites were analyzed by HPLC as described below.

3.8.2 Human recombinant 5-lipoxygenase product formation

For determination of the activity of recombinant 5-LO, partially purified 5-LO (~ 0.5 µg producing ~ 800 -1000 ng 5-LO products in control) was added to 1 ml of a 5-LO reaction mix (PBS, pH 7.4, 1 mM EDTA, 1 mM ATP). After incubation with the test compounds or vehicle (DMSO) for 15 min at 4°C, samples were pre-warmed for 30 s at 37°C and incubated after stimulation with $CaCl_2$ (2 mM) for another 10 min. The addition of 1 ml ice-cold methanol stopped the reaction and 5-LO products formed were analyzed by HPLC as described in the following section (3.8.3). For the experiments with microsomes and phospholipids, 100 µg or 300 µg microsomes or phospholipids were incubated for 15 min on ice with recombinant 5-LO enzyme before test compounds were added for another 15 min on ice and 5-LO product formation was started by adding 2 mM $CaCl_2$ and 20 µM AA (final concentrations)

3.8.3 Solid phase extraction and HPLC analysis of metabolites

After incubation, samples were then diluted with 500 µl ice-cold PBS buffer, 30 µl 1N HCl and 10 µl Prostaglandin $B_1\alpha$ as an internal standard for HPLC analysis. Samples were then either centrifuged (4000xg) or directly subjected to solid phase extraction.Therefore C18-solid-phase-extraction columns (Clean-up® Extraction columns from UTC, Bristol PA, USA) were used. The columns were preconditioned with 1 ml methanol 100 % (v/v) and 1 ml water. After sample application, columns were washed with 1 ml Milli Q water and methanol (25 % v/v). Subsequently, formed 5-LO metabolites were eluted with 300 µl methanol and diluted with 120 µl water. Analysis

of 5-LO products by HPLC allows the detection of leukotriene B_4 (only found in intact cells), its all-trans isomers and 5-HETE, 12-HETE and 15-HETE. Separation of metabolites was carried out on a C18 reversed phase column (Novapak C18 radial column 100mm 5mm I.D.4 µM, Waters) with a mobile phase consisting of 76 % methanol (v/v), 24 % water (v/v) and 0.007 % (v/v) trifluoroacetic acid at a flow of 1.2 ml/min. Products were detected for 30 min, in the first 8 min at a wavelength of 280nm and for the last 22 min at 235nm. In samples from cell preparations, products were expressed as ng/10^6 cells whereas recombinant protein products were described as ng products per amount of protein.

3.9 Phospholipase D activity assay

PLD activity was evaluated using a nonradioactive mass choline assay [317]. Choline quantification is based on choline oxidation by exogenous choline oxidase to betain and H_2O_2. In presence of horseradish peroxidase, the formed H_2O_2 reacts with Amplex-red in a 1:1 stoichiometry to generate the fluorescence product resorufin. Briefly, human PMNL (2.5×10^6 cells) were preincubated with vehicle (0.1 % DMSO), Ssi (30 µM) or the PLD inhibitor 5-fluoro-2-indolyl des-chlorohalopemide (FIPI, Sigma, 100 nM), stimulated with 2.5 µM A23187 (37°C, 3min) and the reaction was stopped on ice. After centrifugation (5 min, 1000×g), cells were suspended in 600 µl of 50 mM Tris-HCl, pH 8 containing 1 mM PMSF and sonicated at 4°C (3 strokes of 5 s, each). Cell disruption was checked by phase contrast microscopy. Samples were centrifuged again (10.000×g, 4°C, 5 min) and supernatants were collected and put on ice. Supernatants (250 µl) were diluted with 250 µl Tris-HCl (50 mM, pH 8) containing 1 mM PMSF. Choline oxidation was performed at 37°C for 1 h after addition of 500 µl Tris-HCl (50mM, pH 8), 100 µM Amplex red, peroxidase (2 units/ml), and choline oxidase (0.2 U/ml). The increase of resorufin fluorescence was monitored using an Aminco-Bowman series 2 spectrofluorometer, at excitation and emission wavelengths of 530 and 590nm, respectively. Choline content was calculated from calibration curves obtained with exogenous choline chloride from 5 to 200 pmol.

3.10 Intracellular calcium mobilization

Freshly isolated PMNL ($1x10^7$ cells in 1 ml PGC buffer) were incubated with 2 µM

Fura-2/AM for 30 min at 37°C. After washing, cells were resuspended in 1 ml PGC buffer, preincubated with Ssi or vehicle (0.1 % DMSO) for 10 min, supplemented with 1 mM $CaCl_2$, and transferred into a thermally controlled (37°C) fluorimeter cuvette in a spectrofluorometer (AB-2) with continuous stirring. Mobilization of intracellular Ca^{2+} was initiated by addition of fMLP (1 µM). Emission wavelength of the fluorescence was 510nm and was measured after excitation at wavelengths 340 and 380nm, respectively. The calculation of intracellular Ca^{2+} levels was based on the method of Grynkiewicz *et al.* [318] where the maximal fluorescence (F_{max}) was generated by cell lysis with 0.5 % Triton-X 100 and minimal fluorescence (F_{min}) by treatment with the Ca^{2+}chelating agent EDTA (10 mM).

3.11 SDS PAGE and Western blot

Aliquots of samples were immediately mixed with the same volume of 2×SDS-PAGE sample loading buffer, heated for 6 min at 95°C, and analyzed for 5-LO protein by SDS-PAGE and Western blotting using the 5-LO anti-serum 1551, AK-7 (raised in rabbit, diluted 1:25) that was kindly provided by Prof. Dr. Olof Rådmark, Stockholm, Sweden. Proteins were separated by SDS-PAGE on a 10 % gel and afterwards blotted onto ECL nitrocellulose membrane (GE Healthcare). After blocking with 2 % ECL advance blocking agent (GE Healthcare) for 1 h at room temperature membranes were incubated with primary antibodies overnight at 4°C. Antibodies were used at 1:1000 dilutions in TBS containing 2 % advance blocking agent. The membranes were then washed and incubated for 1h at room temperature with ECL Plex-CyDye-conjugated antibodies (Cy5-conjugated anti-rabbit, GE Healthcare) diluted 1:2500 in TBS. After washing membranes were dried and then scanned in the Cy5 (excitation filter: 635 nm, emission filter: 680 nm) channels by an Ettan DIGE imaging system.

Target protein	Primary antibody	source	Purchased from
5-LO	AK 7	rabbit	Gift from Karolinska Institutet, Stockholm
ERK 1/2	p42/44 MAPK	rabbit	Cell Signalling
phosphorylated ERK 1/2	Phospho-p42/44 MAPK	rabbit	Cell Signalling

Table 1 *Description of Western blot antibodies*

3.12 Human whole blood assay

For analysis of PG or LT biosynthesis products only freshly withdrawn blood from healthy volunteers was taken and immediately continued processing. The only requirement for the volunteers was no uptake of NSAIDs for at least two days whereas the sex of the volunteers was no knock-out criterion.

3.12.1 Determination of 5-lipoxygenase products in human whole blood

For whole blood assays, freshly withdrawn blood was obtained by venipuncture and collected in monovettes containing 16 IE heparin/ml. Aliquots of 2 ml (A23187) or 3 ml (LPS and fMLP) were pre-incubated with the test compounds or with vehicle (DMSO) for 10 min at 37°C, as indicated, and formation of 5-LO products and 12(S)-hydroxy-5-cis-8,10-trans-heptadecatrienoic acid (12-HHT) was either started by addition of fMLP (1 µM) following priming of blood with 1 µg/ml LPS, or by addition of A23187 (30 µM). The reaction was stopped on ice after 15 (LPS priming and stimulation with fMLP) or 10 (stimulation with A23187) min 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 PGB_1 were added as internal standard. The samples were placed at -20°C for 2 hrs and centrifuged again (600×g, 15 min, 4°C). The supernatants were collected and diluted with 2.5 ml PBS and 75 µl 1 N HCl. Formed 5-LO metabolites and 12-HHT were extracted and analyzed by HPLC as described above.

Alternatively, 450 µl of freshly withdrawn human whole blood from volunteer male and female were incubated with drugs or vehicle over a preincubation period of 30 min at 37°C. By the addition of A23187 (final concentration 20 µM) dissolved in autologous plasma, 5-LO product formation was initiated. The final vehicle (DMSO) concentration was < 1 %, final volume 0.5 ml). After 15 min the reaction was stopped by rapid cooling of the plate on ice. Samples were then centrifuged at 1.000xg at 4°C for 15 min and 5-HETE, LTB4, 12-HETE, 15-HETE, and PGE_2 in the plasma supernatant were analyzed using LC-MS/MS (liquid chromatography coupled with tandem mass spectrometry). LC-MS/MS analysis was performed on an API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Darmstadt, Germany). The mass transitions used were m/z 335.1→195.0 (LTB_4), m/z 319.2→115.0 (5-HETE), m/z 319.2→178.9 (12-HETE), m/z 319.2→219.1 (15-HETE). For internal standardization the following transitions were used: m/z 339.2→196.9 (2H4-LTB_4), m/z 327.2→116.1 (2H8-5-HETE), m/z 327.2→184.1 (2H8-12-HETE), m/z 327.2→ 225.9 (2H8-15-HETE). Linearity of the calibration curve was proven from 0.5 to 2500 ng ml/1 for each eicosanoid. Mean accuracy of the assay was 99.9 +/- 3.25% for LTB_4, 99.85 +/- 4.8% for 5-HETE, 100.2 +/- 4.8% for 12-HETE and 99.76 +/- 4.4% for 15-HETE.

3.12.2 Determination of cyclooxigenase-2 products in human whole blood

Aliquots of heparinized human whole blood (500 µl) were incubated with LPS (10 µg/ml) additionally to the test compound or vehicle (DMSO) for 24 hrs at 37°C. Existing platelet COX-1 activity was eliminated by the addition of aspirin simultaneously with test compounds (100 µM). Plasma was separated by centrifugation at 1.000xg and 4°C for 1 min and PGE_2 in the plasma supernatant was analyzed by LC-MS/MS using the API 4000 triple quadrupole mass spectrometer as described above.

3.13 Determination of human recombinant cyclooxigenase-2 activity

Human recombinant COX-2 activity was assessed by a competitive enzyme-immunoassay with $PGF_{2\alpha}$ as directly measured eicosanoid. COX-2 activity calculation was based on a prostaglandin standard curve. All procedures were according to the manufactors protocoll from Cayman® Chemicals.

3.14 Saturation-transfer-difference nuclear magnetic resonance anlysis of 5-lipoxygenase

Wildtype 5-LO was expressed in *E.coli* and purified by ATP-affinity-chromatography following ion-exchange chromatography (Resource Q 6 ml column, GE Healthcare). For buffer exchange the protein was dialyzed through a regenerated cellulose membrane with a molecular weight cut-off of 3.5 kDa (Spectra/Por 3, Spectrumlabs, Bredam, Netherlands) against deuterated phosphate buffer (50 mM) pH 7.5 over night at 4°C under continous stirring. After dialysis the protein solution was concentrated using membrane filter units (Amicon® Ultra 4, Millipore, Cork, Ireland) where all residual H_2O was finally replaced by D_2O. Protein concentration was detemined by Bradford assay. Samples were incubated with vehicle (DMSO-d6) or sulindac sulfide (dissolved in DMSO-d6) and 4.4-dimethyl-4-silapentane-1-sulfonic acid (DSS) was added for an internal standard. Measurements were performed in a BRUKER Av III-MHz spectrometer with Txi ^{1}H-^{13}C-^{15}N cryo-sample vial. Suppression of the water signal was processed by the excitation sculpting program. Irradiation frequency within the *ON*-resonance experiments was 0.8 ppm, within the *OFF*-resonance experiments 21.4 ppm with 50 Gaussian pulses with 1 % truncation. For suppression of protein signal, 30 ms spinlock was used. Data processing was performed with the program Topsin 2.1 (2009).

3.15 Transfection of HeLa cells and reporter gene assay

24 hrs prior to transfection, HeLa cells were seeded at a density of 4×10^4 cells per well. 0.8 µg 5-lipoxygenase luciferase reporter gene plasmid and 0.2 µg pCMVRenilla as internal standard were transfected using the calcium phosphate method. After transfection and incubation with Ssi (48 hrs), cells were assayed for reporter gene activity with the Dual-Glo™ Stop and Glow Luciferase Assay system following the manufacturer's protocol (Promega) and measured with a Tecan infinite® M200 reader. Renilla activity was used to normalize the luciferase activity to the transfection efficacy. Data are shown as relative light units (RLU).

3.16 Quantitative realtime PCR

MM6-cells were harvested and washed twice with cold PBS buffer, then RNA extraction was perfomred using the total RNA Mini Kit (Omega) and concentrations of RNA were confirmed with a Nanodrop spectrofotometer (Implen GmbH, Munich). 2 µg of total RNA were then reverse transcribed into cDNA with the iScript cDNA synthesis Kit (Biorad). Quantitative PCR analysis was performed using a MyiQ cycler (Biorad). The used 5-LO primers covered the region between -286 to +78kb of the human 5-LO promotor.

Target gene	***Primer forward***	***Primer reversed***	***Temperature [°C]***	***Number of cycles***
5-LO	GTTCCTGAATGGCTGCAAC	GTTCCTGAATGGCTGCAAC	60	32
Sp1	GCTTCAGGCTGTTCCAAACTCT	CTGCCAACTGACCTGTCCATT	60	40
ß-actin	CGGGACCTGACTGACTACCTC	CTTCTCCTTAATGTCACGCACG	60	32

Results were normalized against actin ct values. Each sample was set up in triplicates and amplified for the indicated cycles. The expression was quantified by the comparative DDCT method.

Table 2: *Description of conditions and primers used for quantitative PCR.*

3.17 Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed using the ChIP assay kit (Millipore, Billerica, MA). MM6-cells (1×10^7) were treated with the indicated conditions of Ssi for 72 hrs and incubated with 10 µM of MS-275 16 hrs prior to harvest. After 72 hrs, cells were fixed with 1 % formaldehyde for 10 min at 37°C and further precipitated with antibodies against Sp1 (Millipore) or normal rabbit IgG overnight at 4°C. Purified DNA samples were used as a template for PCR amplification. The region between -286 to +78 of the human 5-LO promoter was amplified for 32 cycles.

3.18 Statistics

All data are presented as mean + standard error of the mean (s.e.m.). GraphPad Prism version 5.0 (GraphPad Software, San Diego, California, USA) was used for statistical analysis. Data were subjected to one-way ANOVA coupled with two-sided turkey's post t-test for multiple comparisons. The IC_{50} values were calculated using GraphPad Prism version 5.0 and a sigmoid concentration-response curve-fitting model with a variable slope.

4 RESULTS

4.1 Inhibition of leukotriene biosynthesis by sulindac sulfide

4.1.1 Effect of Ssi on 5-lipoxygenase product formation in intact PMNL

The effects of sulindac and its metabolites on 5-LO product (LTB_4, its all-trans isomers, and 5-H(p)ETE) formation were analyzed in human PMNL, the major 5-LO product-forming cells in peripheral blood [319]. The first series of experiments utilized A23187 as a stimulus, in order to activate 5-LO directly by an increase in intracellular calcium to circumvent receptor signaling (e.g. receptor mediated MAPK activation leading to phosphorylation of 5-LO). Exogenous AA (2 and 20 µM) as a supplement ensured an ample substrate supply for 5-LO. Both measures were taken to exclude interference of the compounds with signaling pathways and AA release. Concentration-response studies revealed that Ssi potently inhibited 5-LO product formation in A23187-stimulated PMNL in the absence as well as in the presence of exogenously added AA (2 and 20 µM), without significant different potency (IC_{50} = 9 and 10.6 µM).

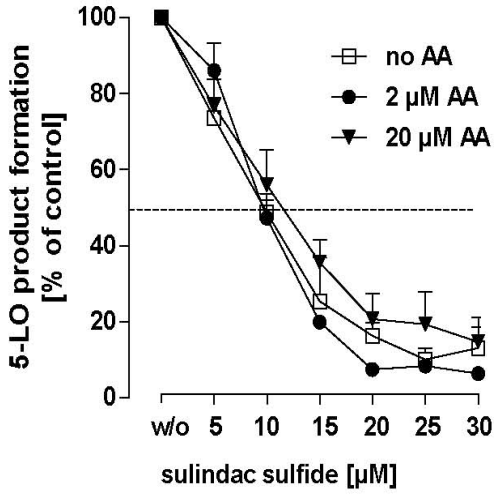


Fig. 12: *Effect of Ssi on 5-LO product formation in A23187-stimulated intact PMNL in the presence or absence of AA*

$7x10^6$ cells were incubated with Ssi in increasing concentrations in the absence or presence of 2µM or 20 µM AA, stimulated with A23187 (2.5 µM) and formed 5-LO metabolites were analyzed by HPLC. n ≥ 3. Values are expressed as mean + SEM.

Beside Ssi, the related compounds sulindac (Sul) and indomethacin (Ind) which displays a structural analogue to Ssi, were tested for 5-LO inhibition. Sulindac sulfone (Sso), a metabolite of sulindac, was investigated to test the specificity of suppression of 5-LO by Ssi. The compounds did not show any inhibiton of 5-LO product formation (Fig. 13). Only the well-known potent 5-LO inhibitor BWA4C (BWA), which is an iron-ligand inhibitor (refer to Fig. 11, section 1.2.9) shows 5-LO inhibition and was used as control.

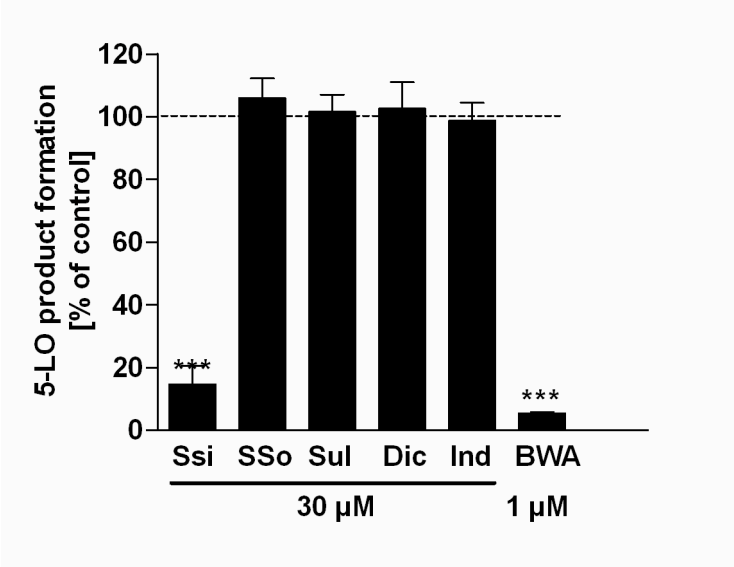


Fig. 13: *5-LO inhibitory effect of Ssi, its metabolites and related compounds in A23187-stimulated PMNL*

*$7x10^6$ cells were stimulated with A23187 (2.5 µM) and AA (20 µM) after incubation with compounds (30 µM) for 10 min at 37°C. Synthesized 5-LO products were detected by HPLC. n ≥ 3. ***, p ≤ 0.001. Values are expressed as mean + SEM.*

4.1.2 Effect of Ssi on 5-, 12- and 15-LO product formation

Since PMNL preparations (purity >95 %) contain eosinophils expressing 15-LO-1, also the effects of Ssi on the formation of 15-H(p)ETE were analyzed. In contrast to 5-LO product synthesis, the formation of 15-H(p)ETE was slightly increased, rather than inhibited at SSi concentrations > 10 µM (Fig. 14). In addition, the effect of Ssi on platelet-type 12-LO was investigated. 12-H(p)ETE product formation was elicited in platelets by 5 µM AA, but no inhibition by Ssi was seen up to 30 µM. In contrast, activation of 12-LO could be detected. Ssi therefore selectively inhibited cellular 5-LO, but not 12- or 15-LO.

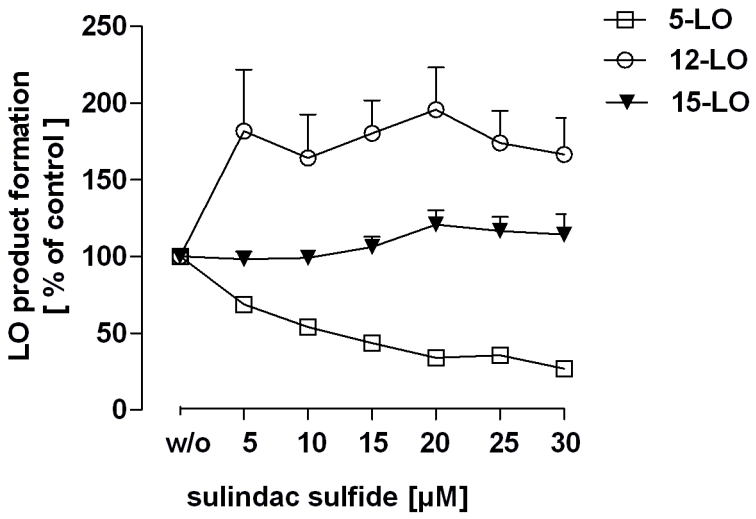


Fig. 14: *Effect of Ssi on 5-, 12- and 15-LO product formation*

For analysis of 12-LO product formation, freshly isolated platelets were incubated with 5 µM AA whereas 5- and 15-LO were analyzed in PMNL preparations. 7x10^6 cells were stimulated with 2.5 µM A2318 supplemented with 20 µM AA. All formed LO metabolites were detected by HPLC. n ≥ 3. Values are expressed as mean + SEM.

4.1.3 Effect of Ssi on 5-lipoxygenase product formation of intact PMNL triggered by different stimuli

Previous studies showed that the type of stimulus and the signal transduction pathways mediating 5-LO activation can lead to different efficacies of 5-LO inhibitors. Therefore, different stimuli were tested to evaluate the 5-LO inhibition pattern of Ssi.

Effect of Ssi on 5-lipoxygenase actvitiy in intact PMNL stimulated with physiological stimuli

In another series of experiments, PMNL were primed with lipopolysaccharide (LPS) in the presence of adenosine deaminase (Ada) and then challenged by the natural chemoattractant fMLP. These assay conditions were designed to closely mimic pathophysiological conditions in the body [319] and therefore offer an estimate of the efficacy of 5-LO inhibitors *in vivo* [308]**.** Generation of 5-LO products in PMNL following stimulation with fMLP (after priming with LPS/Ada, 1 µg/ml/0.2 U/ml) was potently suppressed by Ssi with an IC_{50} = 7.7 µM (Fig. 15). (Data obtained in cooperation with C.Pergola, group of Prof. O. Werz, University of Tübingen)

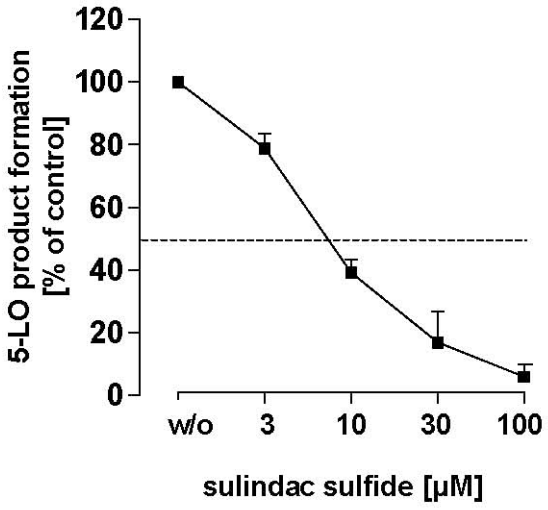


Fig. 15: *Effect of Ssi on 5-LO product formation in intact PMNL triggered by LPS/Ada and fMLP*

Freshly isolated PMNL (7x10^6 cells) were first primed with 1 µg/ml LPS and Ada (0.2 U/ml) for 30 min and then incubated with 1 µM fMLP for another 15 min. Quantification of 5-LO products was carried out by HPLC analysis. n ≥ 3. Values are expressed as mean + SEM.

Effect of Ssi on 5-lipoxygenase actvitiy in intact PMNL stimulated with chemical stimuli

Previous studies showed that the type of stimulus and the signal transduction pathways mediating 5-LO activation can lead to different efficacies of 5-LO inhibitors in PMNL [320]. Hence, we compared the potencies of Ssi in PMNL stimulated with A23187 (2.5 µM), hyperosmotic shock (0.3 M NaCl) and genotoxic stress using sodium arsenite [321]. All incubations contained exogenous AA (20 µM). IC_{50} values ranged between 8.6 and 14 µM (Fig. 16**)**, but in contrast to A23187-activated cells, Ssi failed to completely reduce 5-LO product synthesis in PMNL challenged by cell stress, plateauing with approximately 30 % remaining 5-LO activity.

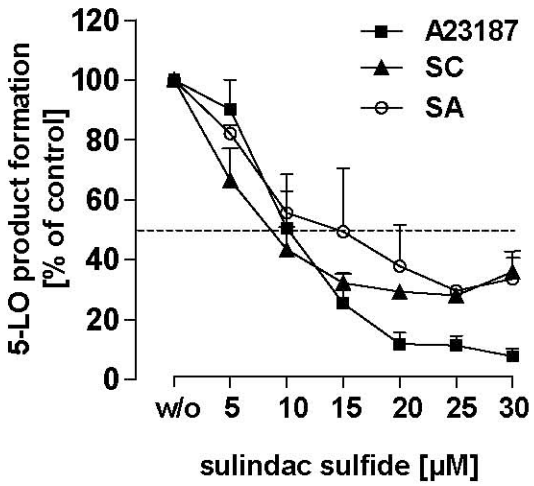


Fig. 16: *Effect of Ssi on 5-LO product formation in PMNL stimulated with A23187, SC and SA*

5x10^7 cells were pre-incubated with Ssi in increasing concentrations for 15 min at 37°C. SA (10 µM) and SC (300 mM) were added 3 min prior to the addition of 20 µM AA, ionophore was added together with 20 µM AA. 5-LO metabolites were quantified by HPLC analysis. n=3. Values are expressed as mean + SEM.

4.1.4 Effect of sulindac and metabolites on human recombinant 5-lipoxygenase product formation

As Ssi potently reduced 5-LO product formation in intact PMNL the question was addressed whether Ssi interferes directly with the catalytic domain of the 5-LO enzyme. Therefore, affinity-purified human recombinant 5-LO protein was incubated with the compounds indicated and 20 µM AA was added as substrate. Sulindac, which underlines oxidative metabolism in the human body to either sulindacsulfone and further conversion to Ssi, showed no suppressive effects whereas sulindacsulfone moderately, but not significantly reduced 5-LO activity by ~ 20 %. Only treatment with Ssi led to a potent inhibition of 5-LO enzyme activity with an IC_{50} of 20 µM (Fig. 17) although with a somewhat lower potency compared to intact PMNL (IC_{50} values 7.7 – 14 µM).

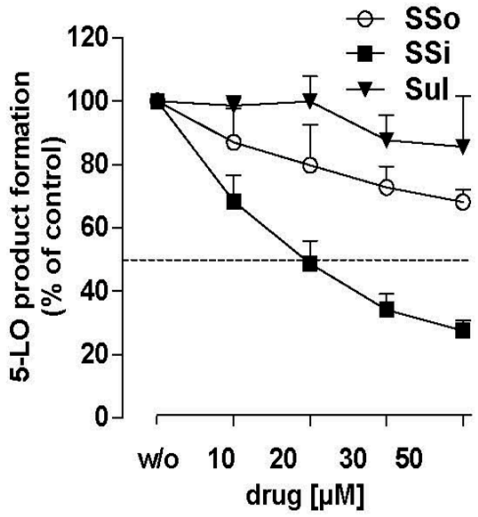


Fig. 17: *Effect of Sul and metabolites Sso and Ssi on recombinant 5-LO product formation*

Recombinant 5-LO was preincubated for 15 min with the compounds as indicated and then stimulated with 2 mM Ca^{2+} and 20 µM AA was added. After the reaction 5-LO HPLC analysis of 5-LO products followed. n=4. Values are expressed as mean + SEM.

But in contrast to BWA4C, Ssi did not entirely reduce 5-LO activity in the cell-free assay and about 30 % residual activity still remained. BWA4C was used as reference 5-LO inhibitor. Indomethacin (Ind), an unselective COX-inhibitor, was used as negative control. As expected, Ind did not influence 5-LO product formation in contrast to BWA4C. Sso had only weak inhibiting effects (Fig. 18).

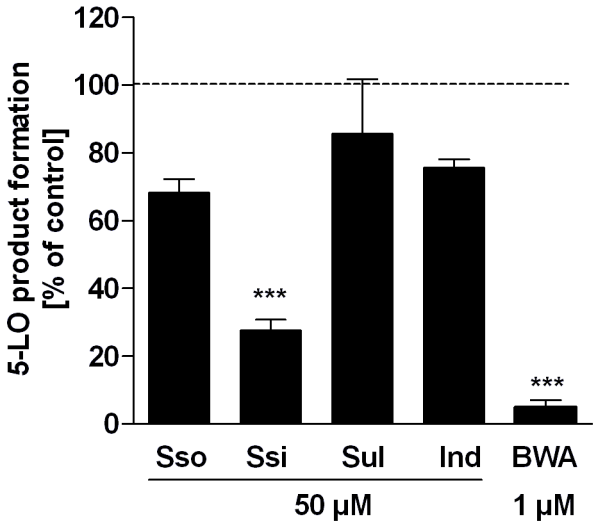


Fig. 18: *Effect of Ssi, Sso, sulindac, indomethacin and BWA4C at the concentrations indicated on recombinant 5-LO activity*

*5-LO product formation was determined by HPLC after incubation with Sul, Sso, Ssi and controls BWA4C and indomethacin and stimulation of recombinant 5-LO protein with Ca^{2+} (2 mM) and AA (20 µM). n ≥ 3. ***, p ≤ 0.001. Values are expressed as mean + SEM.*

4.1.5 Effect of Ssi on A23187-induced subcellular redistribution of 5-lipoxygenase in intact PMNL

Since Ssi was more effective in the context of intact PMNL than with purified recombinant 5-LO, the inhibitor may affect additional 5-LO stimulatory mechanisms in intact cells, such as FLAP. In fact, sulindac exhibits structural similarity to the FLAP inhibitor MK-886 (refer to 1.2.9). Functional FLAP acts as an anchor for facilitating access to the natural substrate AA and FLAP-inhibiting compounds block the translocation to the nuclear membrane. FLAP inhibitors share the ability to reverse agonist-induced translocation of 5-LO from the cytosol to the nuclear envelope in PMNL [322]. Ssi (10 -100 µM), but not Sso or Sul, partially reversed 5-LO redistribution from the cytosol to the nucleus in PMNL evoked by A23187 with an efficacy comparable to MK-886 (100 nM). As observed in previous experiments, the 5-LO inhibitor BWA4C did not affect A23187-induced 5-LO redistribution at a concentration of 300 nM [309]. (Data obtained in cooperation with C.Pergola, group of Prof. O. Werz, University of Tübingen)

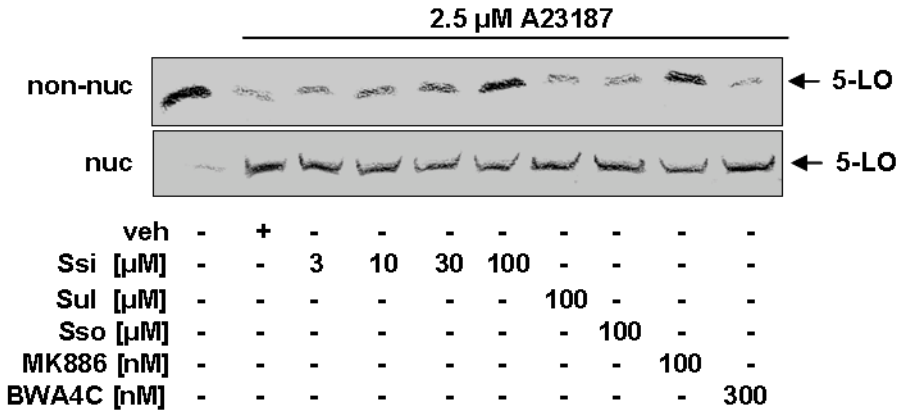


Fig. 19: *Effect if Ssi and other control agents on A23187-induced subcellular redistribution of 5-LO in intact PMNL*

$3x10^7$ cells were pretreated with the compounds at 37°C for 15 min. A23187 (2.5 µM) were added for another 5 min. Nuclear (nuc) and nonnuclear (non-nuc) fractions were obtained by 0.1% NP-40-lysis and samples were then subjected to SDS PAGE and Western blotting. A representative out of three independent experiments is shown.

4.1.6 Effect of A23187-induced 5-lipoxygenase product formation in PMNL in the presence of MK-886

To further understand the mode of action of Ssi on 5-LO and to exclude any FLAP-inhibiting properties of the drug, a competition assay was designed where Ssi's ability to suppress cellular 5-LO product formation in an additive manner to MK 886 was investigated. PMNL were incubated with 5 µM MK-886 and subsequently stimulated with a mixture of 2.5 µM A23187 and 40 µM AA. In the presence of ample exogenous AA (40 µM), the effectiveness of FLAP inhibitors is typically reduced and even high concentrations of MK-886 (5 µM) failed to completely inhibit 5-LO product formation, and approximately 45% 5-LO activity still remained [323]. This basal activity is considered a FLAP-independent conversion of AA by 5-LO. In line, the failure of coincubation of MK-886 with the FLAP inhibitor MK-0591 (IC_{50}=3.1 nM) to further reduce 5-LO product formation demonstrated this independence [324]. By contrast, co-incubation of Ssi (10 µM) together with MK-886 further suppressed 5-LO product synthesis suggesting that Ssi has molecular targets other than FLAP. Co-incubation of

MK-886 with zileuton, a well-known direct 5-LO inhibitor, also reduced activity to a near zero level.

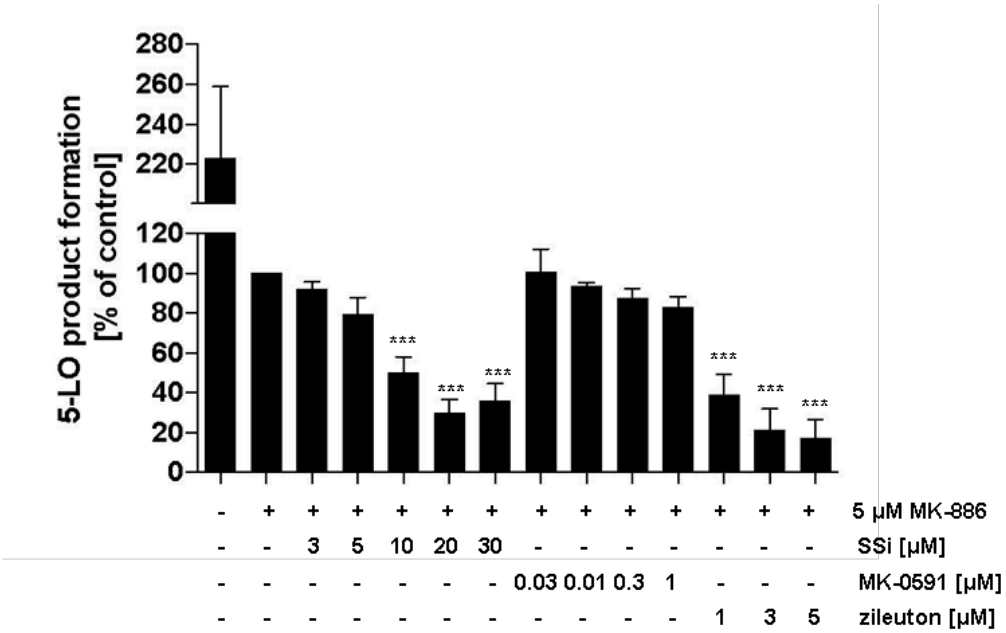


Fig. 20: *Effect of Ssi and related compounds on A23187-induced 5-LO product formation in PMNL in presence of MK-886*

*Cells were preincubated with MK-886 (5µM) and other compounds for 10min and the reaction was started by the addition of 40µM AA and 2.5µM A23187. Activity was assessed by HPLC analysis and expressed relatively to vehicle control (100%). n=3-6. *** p ≤ 0.001; * p≤ 0.05. Values are expressed as mean + SEM.*

4.1.7 Suppression of 5-lipoxygenase product formation by Ssi in human whole blood

Finally, Ssi was tested for its ability to suppress LT formation in human whole blood. Several inhibitors of LT biosynthesis have demonstrated high potency in isolated leukocytes but failed to efficiently interfere with 5-LO product formation in whole blood, due to plasma protein binding or to competition with other molecules, such as fatty acids [146] . Thus, the ability of a given compound to suppress LT formation in the whole blood assay may provide some perspective of its efficacy *in vivo*. Ssi was therefore added to venous blood from healthy volunteers to concentrations achieved under standard oral therapy with sulindac (up to 40 µM [60]). After 10 min, the blood

was either pre-incubated with LPS for 30 min and then stimulated with 1 µM fMLP or stimulated with the Ca^{2+}-ionophore A23187 alone. The formation of 5-LO products was concentration-dependently inhibited by Ssi with IC_{50} values of 18.7 (fMLP) and 41.4 µM (A23187) (Fig. 21). Furthermore, the formation of 12-H(p)ETE in the same samples of A23187-challenged blood was only moderately inhibited (34.5 ± 14% at 100 µM SSi, data not shown). (Data obtained in cooperation with C.Pergola, group of Prof. O. Werz, University of Tübingen; LC-MS/MS measurements were performed by C. Angioni, group of Prof. G. Geißlinger, Hospital of Goethe University, Frankfurt)

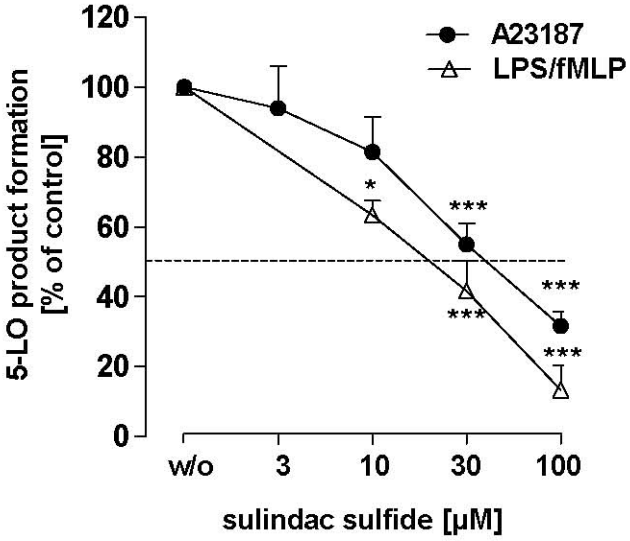


Fig.21: *Effect of Ssi on 5-LO product formation triggered by different stimuli in human whole blood*

*Human whole blood was incubated with LPS (1 µg/ml) and fMLP (1 µM) or A23187 (30 µM) and analysed by LC-MS/MS for 5-LO metabolites. n ≥ 3. *** p ≤ 0.001; * p ≤ 0.05. Values are expressed as mean + SEM.*

Both sulindac and Sso failed to significantly inhibit 5-LO product formation at concentrations up to 100 µM, whereas the 5-LO inhibitor BWA4C (3 µM; control) did suppress 5-LO (Fig. 22). (LC-MS/MS measurements were performed by C. Angioni, group of Prof. G. Geißlinger, Hospital of Goethe University, Frankfurt)

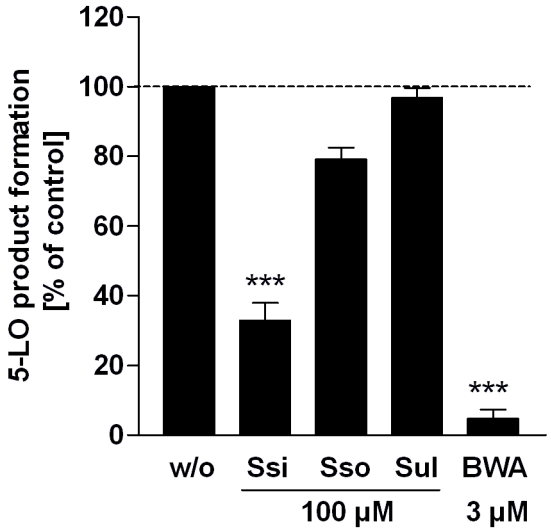


Fig. 22: *Inhibitory effect of Ssi, Sso, Sulindac and BWA4C on 5-LO product formation in human whole blood*

*Human whole blood was incubated with the compounds as indicated and the stimulated with A23187 (30 µM) for 15min and analyzed for 5-LO metabolites by LC-MS-MS. n ≥ 3. *** p ≤ 0.001; *p ≤ 0.05. Values are expressed as mean + SEM.*

4.1.8 Suppression of cyclooxigenase product formation by Ssi in human whole blood

Previous studies attributed Ssi's anti-inflammatory effect to the inhibition of prostaglandin synthesizing enzymes, the COX enzymes [325]. In order to compare the inhibiting properties of Ssi towards 5-LO and COX enzymes, COX-1 and COX-2 activity was evaluated in whole blood. Therefore, blood samples were incubated with drugs and stimulated with LPS 1 µg/ml and incubated 24 hrs at 37°C. Prostanoids were then subjected to LC-MS/MS analysis. Lipid mediators analyzed were the COX-2 derived PGE_2, the mainly via the COX-1 pathway obtained 12-HHT and the 5-LO derived LTB_4 and 5-HETE. Products formed via the COX-pathways are effectively suppressed so that PGE_2 and 12-HHT production reached almost zero level at Ssi concentrations of 100 µM. Calculated IC_{50} values range from 3 µM (12-HHT) to 3.9 µM (PGE_2). 5-LO activity was affected as well and inhibited at 100 µM Ssi to ~20% remaining activity (Fig. 23). (COX-1 derived products were measured by C.Pergola, group of Prof. O. Werz, University of Tübingen; LC-MS/MS measurements of COX-2

and 5-LO products were performed by C. Angioni, group of Prof. G. Geißlinger, Hospital of Goethe University, Frankfurt)

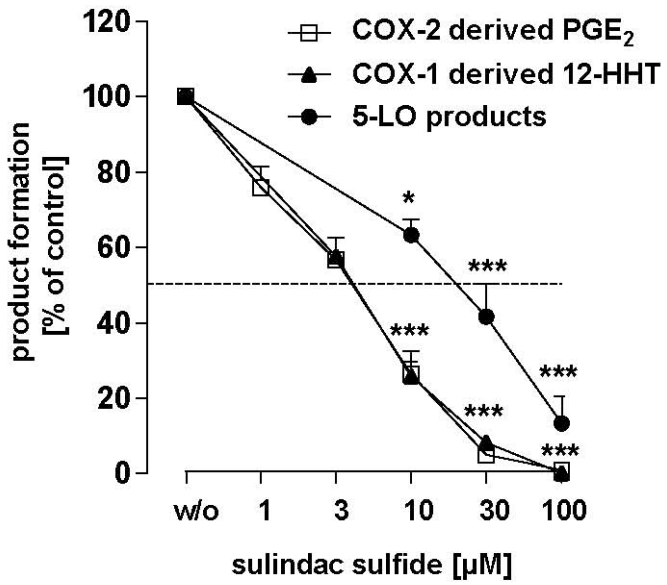


Fig. 23: *Effect of Ssi on eicosanoid formation in human whole blood*

*Ssi was added to whole blood in concentrations as indicated. Samples were either stimulated with LPS (for 24 hrs) for prostanoid analyses or with A23187 (30 µM) and incubated for 15 min for LT analyses. n ≥ 3. *** p ≤ 0.001; * p ≤ 0.05. Values are expressed as mean + SEM.*

Additionally, influence of the prodrug Sulindac and the metabolites Sso and Ssi on COX-2 derived PGE_2 and COX-1 derived 12-HHT product formation was assessed in human whole blood. COX-product formation was not or only moderately influenced by Sul and SSo which is in line with the reported lack of inhibition of COX by these molecules described in section 4.1 and 4.2. In contrast, Ssi potently suppressed the formation of metabolites to almost zero levels (Fig. 24). (COX-1 derived products were measured by C.Pergola, group of Prof. O. Werz, University of Tübingen; LC-MS/MS measurements were performed by C. Angioni, group of Prof. G. Geißlinger, Hospital of Goethe University, Frankfurt)

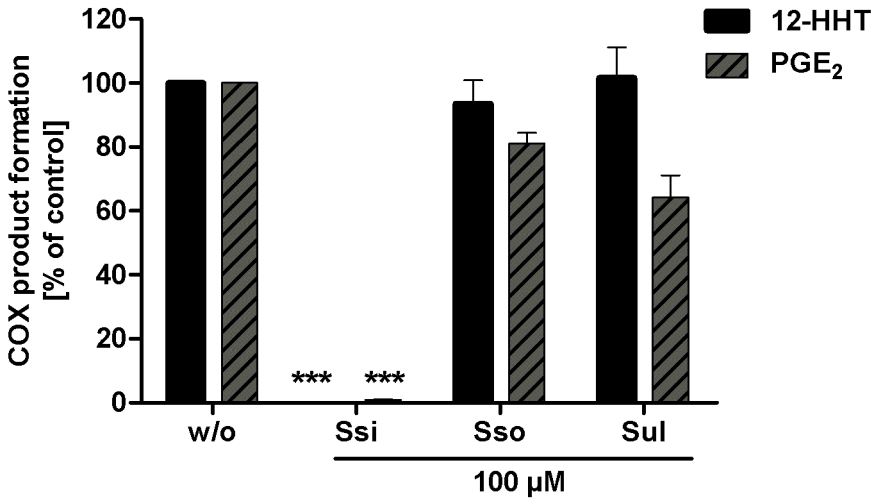


Fig. 24: *Comparison of inhibition of 12-HHT and PGE_2 synthesis by Ssi, Sso and sulindac in human whole blood*

*Human whole blood was incubated with the inhibitors LPS (1 µg/ml) and either stimulated with A23187 (30 µM) and analyzed for 5-LO metabolites by LC-MS-MS. n ≥ 3. *** p ≤ 0.001; * p ≤ 0.05. Values are expressed as mean + SEM.*

4.1.9 Saturation-transfer-difference nuclear magnetic resonance analysis of 5-lipoxygenase in the presence of Ssi

An approach to detect a direct binding of Ssi to 5-LO was undertaken by saturation-transfer-difference nuclear magnetic resonance (STD-NMR) spectroscopy. 5-LO protein produced in *E.coli* was purified first via ATP-affinity chromatography and in a second step via ion-exchange chromatography. For gaining the reference spectrum (^{1}H-NMR spectrum) of Ssi, 160 – 200 µM Ssi were mixed with the additional volume deuterated sodium-dihydrogenphosphate buffer to a final volume of 600 µl in a 3 mm NMR-sample vial. For the STD-NMR sample spectrum, Ssi was added to a 8 µM 5-LO protein solution in deuterated buffer. To ensure protein signals, a protein solution without inhibitor but with DMSO-d6 as solvent was measured (data not shown). A summary of the obtained data is shown in Fig. 25 below. In red, the inhibitor reference spectrum revealed clearly the different signals from the Ssi molecule. Numbers

on the chemical structure of Ssi pointed out the characteristic peaks appearing in the spectrum. Residual peaks are artefacts of the difference formation process. The blue spectrum which presents the STD-NMR spectrum shows significantly reduced peak intensities due to protein binding. Peaks could clearly be allocated to the inhibitor profile in the red spectrum. (Data obtained in cooperation with F. Löhr, group of Prof. V. Dötsch, Goethe University Frankfurt)

STD-NMR spectrum of inhibitor Ssi and inhibitor Ssi with 5-LO protein

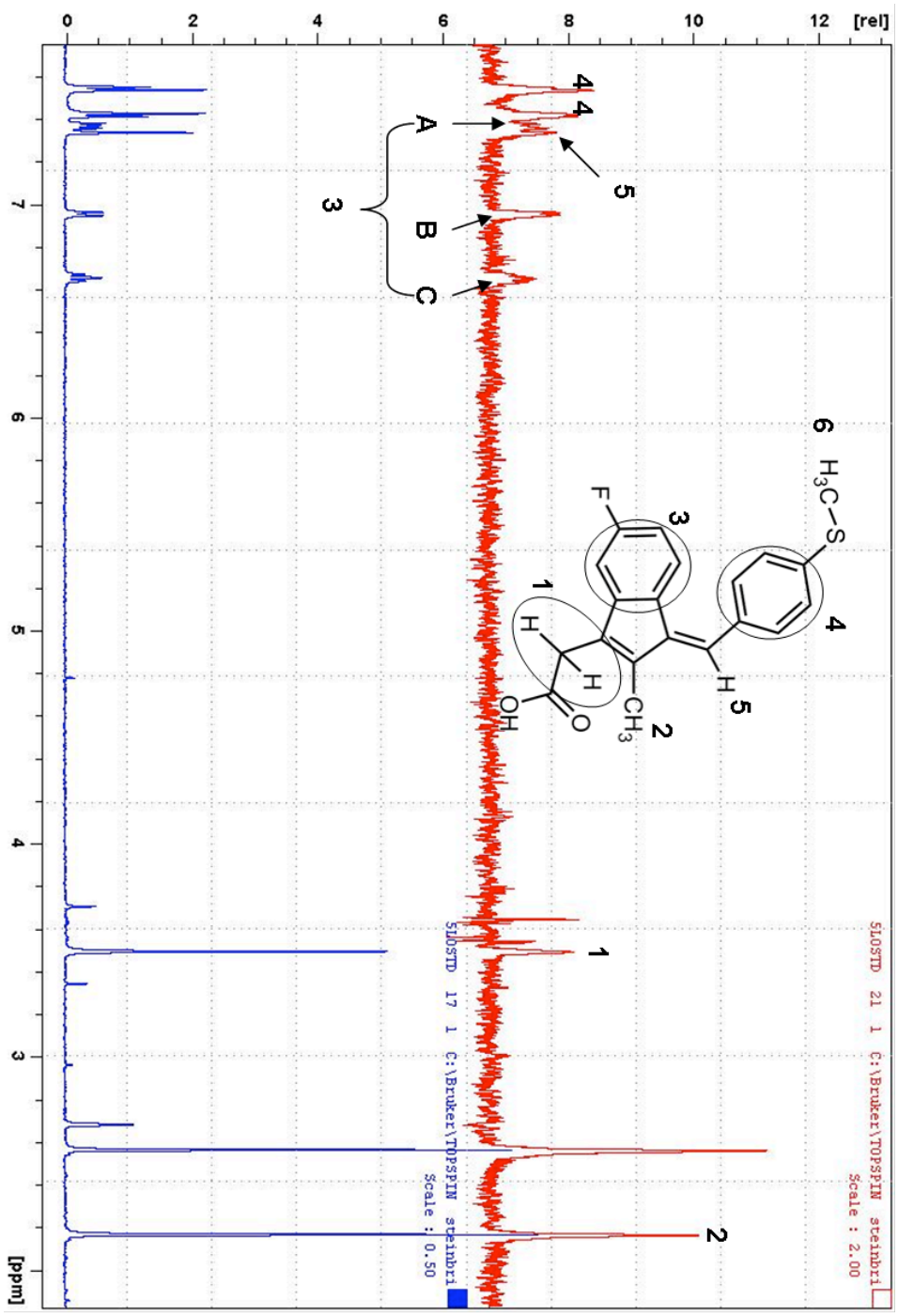


Fig. 25: *STD-NMR spectrum of Ssi and 5-LO in comparison to a ^{1}H-NMR spectrum of Ssi*

The red spectrum displays the reference spectrum of the 5-LO inhibitor Ssi (160 - 200 µM, in deuterated 50 mM phosphate buffer). In blue, the spectrum of the inhibitor in the mixture with 5-LO protein (8 µM, Ssi 160 - 200 µM)) is depicted.

4.2 C2-like domain mediated 5-lipoxygenase inhibition by sulindac sulfide

After description and characterization of Ssi-mediated inhibition of LT biosynthesis in different systems such as intact leukocytes, recombinant purified 5-LO and human whole blood, this chapter furthermore deals with the characterization of the underlying mechanisms of Ssi-mediated suppression of 5-LO product formation. Results of the antecedent section pointed out that reduced 5-LO activity by Ssi did not result from suppression of FLAP or competition with its natural substrate AA. Hence, further investigations focused on the molecular mechanism possibly mediating the interference of the drug with a functional part of the 5-LO enzyme.

4.2.1 Comparison of the 5-lipoxygenase inhibitory potency of Ssi in different cellular systems

First, it was investigated whether the Ssi's inhibitory potency is influenced by the type of test systems and if the cellular environment and its specific composition impact the efficacy of Ssi's inhibition on LT biosynthesis. Therefore, different cellular preparations of PMNL beside intact cells, such as cell homogenates and the 100,000xg supernatant (S100) were used. 5-LO product formation was started by the addition of $CaCl_2$ (2 mM) and exogenously added AA (20 µM). The concentration-response curves in Fig. 26 revealed a considerable difference in Ssi's potency to suppress 5-LO product formation comparing intact cells and the purified recombinant enzyme versus broken cell preparations and the S100. Both, intact PMNL and pure 5-LO showed IC_{50} values ranging from ~ 15-25 µM in contrast to broken cell preparations and the S100 where a clear loss of potency of Ssi is detectable. This loss of potency is reflected in IC_{50} values from ~ 30 µM (S100) up to ~ 100 µM (cell homogenates).

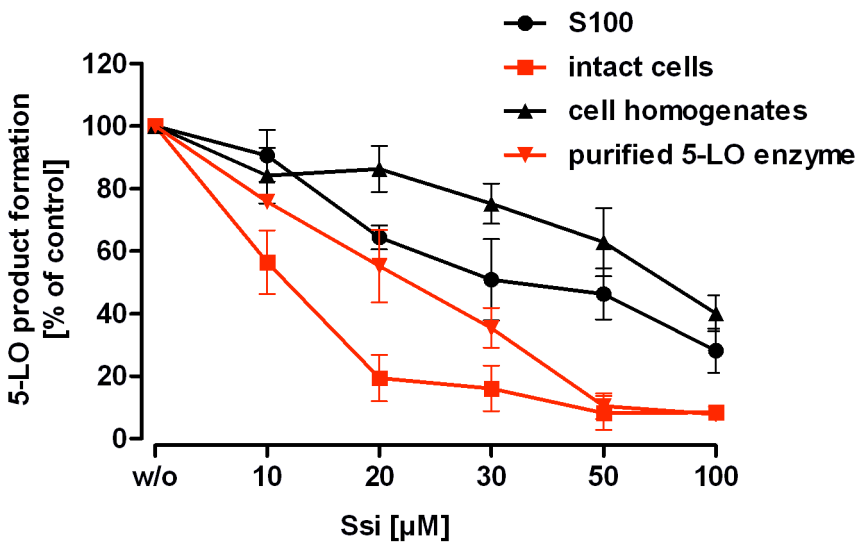


Fig. 26: *Comparison of Ssi's 5-LO inhibitory potency in intact PMNL, cell homogenates, S100 and with purified 5-LO enzyme*

5-LO activity was measured in intact cells (7x10^6) stimulated with 2.5 µM A23187 and 20 µM AA. In PMNL homogenates, S100 and recombinant enzyme, 5-LO product formation was initiated by the addition of 2 mM Ca^{2+} and 20 µM AA. Levels of 5-LO metabolites were assessed by HPLC analysis. n=4. Values are expressed as mean + SEM.

Changes of the cellular composition of different cellular systems may interfere with the redox tone within the catalytic unit of the 5-LO enzyme which contains a non-heme iron that needs to be transformed to the ferric state (Fe^{3+}) during enzyme activation. Thus, it seemed obvious to evaluate whether the observed loss of potency of Ssi in homogenates and S100 depends on a modified oxidation state of the enzyme as true for non-redox type 5-LO inhibitors [289]. Therefore, intact cells and cell preparations were incubated with Ssi or additionally with the reducing agent DTT (5 mM). ZD-2138, a non-redox-type inhibitor (refer to Fig. 11, section 1.2.9) which inhibits 5-LO potently in Ca-ionophore stimulated intact PMNL (IC_{50} ~ 50 nM) and which was reported to lose its 5-LO inhibitory potency in cell-free preparations under non-reducing conditions, functioned as internal assay control (Fig. 28) [289, 326]. Inhibition of 5-LO product formation by Ssi occurred in intact cells whereas Ssi did not suppress 5-LO product formation in homogenates and S100 regardless of the presence or absence of DTT resulting in only ~30 % enzyme inhibition. BWA4C worked as control agent and led to ~ 80 % enzyme inhibition (Fig. 27).

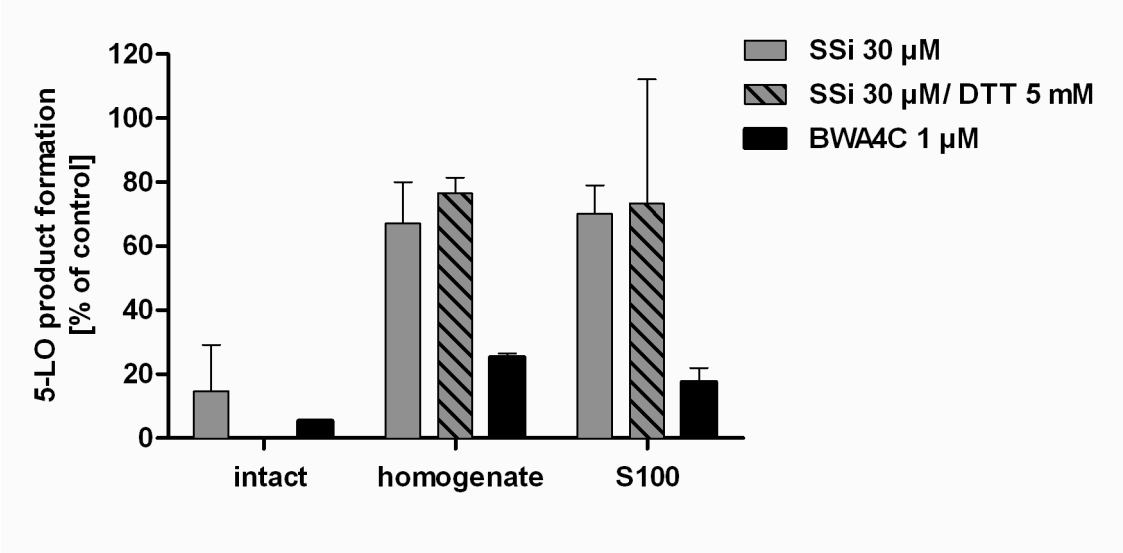


Fig. 27*: Effect of Ssi and BWA4C on 5-LO product formation in intact cells, homogenates and S-100 in presence or absence of DTT*

Intact cells (7x10^6) and cell preparations (homogenates and S100) were either incubated with Ssi alone or in combination with 5 mM DTT, stimulated with Ca-ionophore (2.5 µM, intact cells) or Ca^{2+} (2 mM, homogenates and S100) and AA (20 µM) was supplemented. HPLC analysis for product formation analysis was performed.n=4. Values are expressed as mean + SEM.

In intact cells, ZD-2138 led to suppression of 5-LO product formation regardless of whether DTT was added to the reaction mixture or not. Contrarily, in cell homogenates and S100 its inhibitory potency on 5-LO enzyme was depending on the presence of DTT and was only effective in DTT-containing samples.

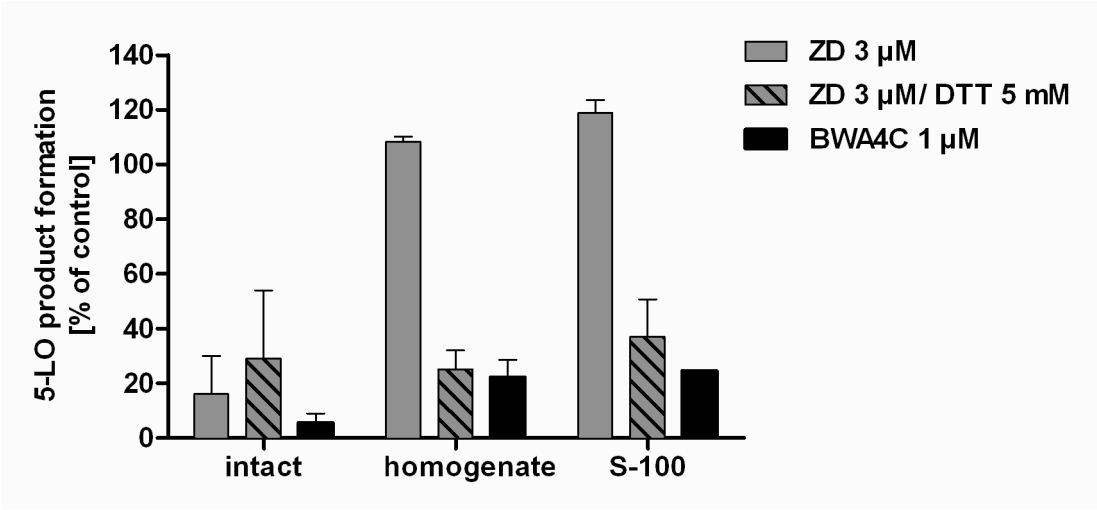


Fig. 28 *Effect of ZD-2138 and BWA4C on 5-LO product formation in intact cells, homogenates and S-100 in presence or absence of DTT*

5-LO activity was measured in intact cells ($7x10^6$), stimulated with 2.5 µM A23187 and 20 µM AA. In PMNL homogenates, S100 and recombinant enzyme, 5-LO product formation was started by the addition of 2 mM Ca^{2+} and 20 µM AA. Levels of 5-LO metabolites were assessed by HPLC analysis. n=4. Values are expressed as mean + SEM.

4.2.2 Influence of Ssi on phospholipase D activity

Further studies were undertaken in order to clarify possible cellular pathways involved in 5-LO modulation mediated by Ssi. The question was addressed whether Ssi shows PLD-inhibiting properties. Cells were preincubated with vehicle (DMSO), Ssi 30 µM, 5-fluoro-2-indolyl-des-chlorohalopemid (FIPI, 100 µM), a potent PLD inhibitor acting as assay control. Product formation was initiated by 2.5 µM A23187 for 3 min. After resuspension, sonication and centrifugation of the cells the produced choline was continued processing and finally resorufin was detected by fluorescence measurements. Results were expressed in three different patterns: choline amount in pmol/mio cells, the change of choline in pmol/mio cells and the choline change as percentage of control. Neither A23187-stimulated cells nor cells treated with 30 µM Ssi, which showed 2-fold excess choline amount over control, were affected compared to the control inhibitor FIPI which inhibits PLD as expected. The relative amount of choline decreased to ~ 70 pmol/mio cells whereas cells incubated with Ssi only showed moderate loss of choline amount which is in line with the percentual depicture (Fig. 29). (Data obtained in cooperation with C.Pergola, group of Prof. O. Werz, University of Tübingen)

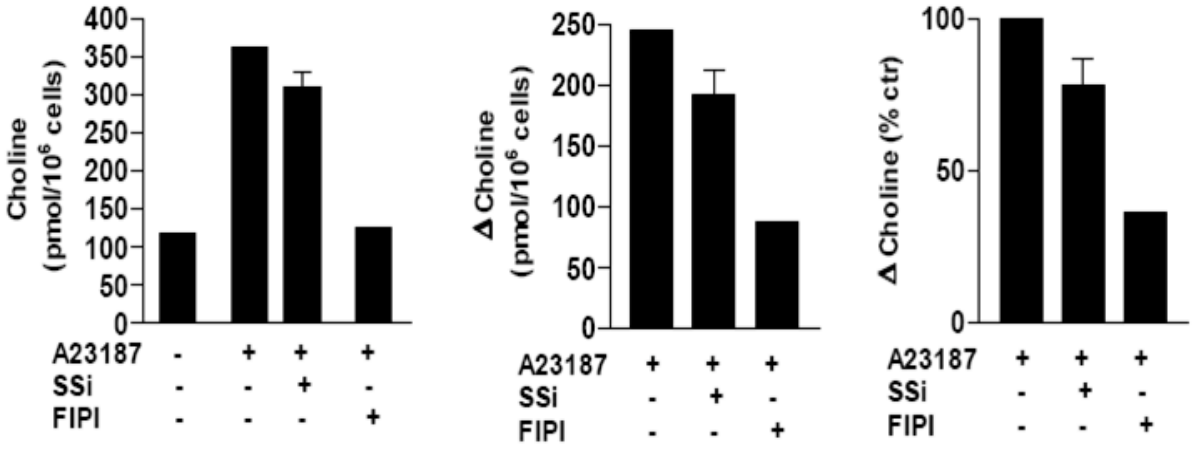


Fig. 29: *Influence of Ssi and FIPI in A23187-stimulated PMNL on PLD activity*

2.5x10^6 cells (PMNL) were incubated with vehicle or Ssi (30 µM) and stimulated with A23187 (2.5 µM) for 3 min. Formed choline was processed to obtain the fluorescent product resorufin which was finally determined. n=3. Values are expressed as mean + SEM.

4.2.3 Effect of Ssi on calcium mobilization in intact PMNL

Increased Ca^{2+} influx triggered by inflammatory stimuli leads to high intracellular Ca^{2+} levels and is a potent stimulatory factor for the enhancement of 5-LO activity [145]. To clarify, whether Ssi shows interactions with a cellular target or is involved in cellular processes upstream of direct interactions with the 5-LO protein, a possible interference with intracellular Ca^{2+} mobilization was investigated. The Ca^{2+} release from human PMNL was observed and detected over a time-course of 100 s. Cells ($1x10^7$) were supplemented with 1 mM $CaCl_2$ and the startpoint of Ca^{2+} release was the addition of 1 µM fMLP known as a less vigorous but physiological Ca^{2+}-related stimulus [327]. As notable in Fig. 30, the intracellular free Ca^{2+} levels rose upon fMLP stimulation after 25 s and slowly decreased reaching ~ 100 nm intracellular Ca^{2+} after 100 s. The sample incubated with Ssi behaved similar to the untreated vehicle (DMSO) control. In the right graph the same picture evoked as it differs only from the depiction where the emission at 530 nm wavelength is plotted against time. Obviously, Ssi

showed no different behaviour compared to control. (Data obtained in cooperation with C.Pergola, group of Prof. O. Werz, University of Tübingen)

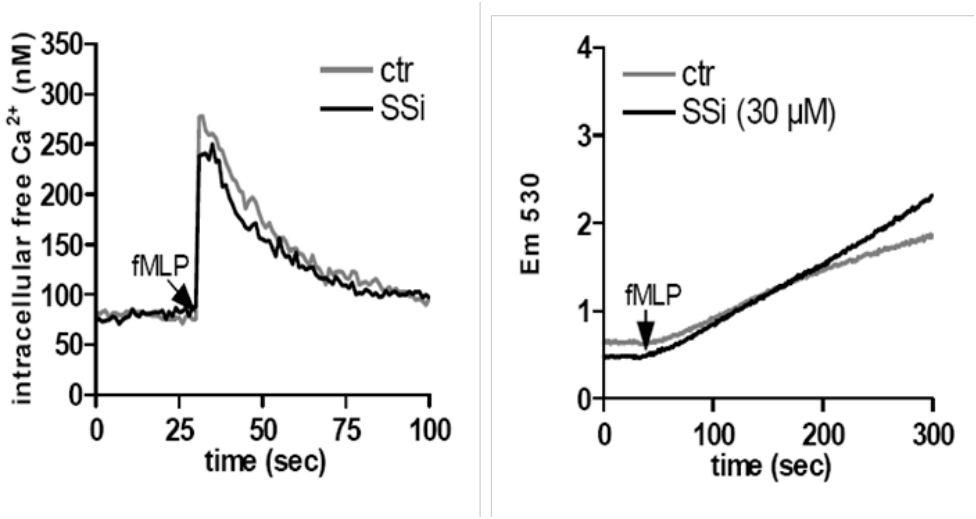


Fig. 30: *Interference of Ssi with calcium mobilization in intact PMNL evoked by the stimulus fMLP*

Free Ca^{2+}-levels were assessed with a fluorospectrometric assay. Incubation of $1x10^7$ intact PMNL with a fluorophor containing substrate for 30 min was performed. After washing the cells, preincubation with either DSMO or Ssi for 10 min at 37°C occurred before the Ca^{2+} release was started with the addition of 1 µM fMLP. Emission was measured at 530 nm with a fluorospectrometer.

4.2.4 Involvement of mitogen-activated protein kinase pathway on Ssi-mediated 5-lipoxygenase suppression in intact PMNL

As kinase pathways play a regulatory role on cellular LT biosynthesis, one reasonable approach seemed to evaluate involvement of kinase pathways in Ssi-mediated 5-LO suppression. It is published that ERK 1/2, a member of the MAP kinase family, phosphorylates the 5-LO protein at residue ser-663 and in turn upregulates the enzyme's activity [190]. To achieve insight into possible Ssi-mediated ERK regulation in intact PMNL and in turn resulting in downstream modulation of 5-LO activity, cells were incubated with Ssi and its metabolites, respectively. U0126 is a potent MK-2 and MK-3 kinase inhibitor that inhibits the phosphorylation reaction of ERK1/2 and therefore displays the inhibitor control. After stimulation of LT product formation the reaction was stopped and cells were mixed with 5x loading buffer (5xLB), boiled and afterwards proteins were separated by SDS-PAGE and Western blot was performed. Increasing concentrations of the active metabolite of Sul, Ssi, did not alter the level of

phosphorylated ERK-2 protein as demonstrated in Fig. 31. Likewise, Sul and Sso showed the same results. As expected, the control inhibitor U0126 assay inhibited the phosphorylation of ERK. (Data obtained in cooperation with C.Pergola, group of Prof. O. Werz, University of Tübingen)

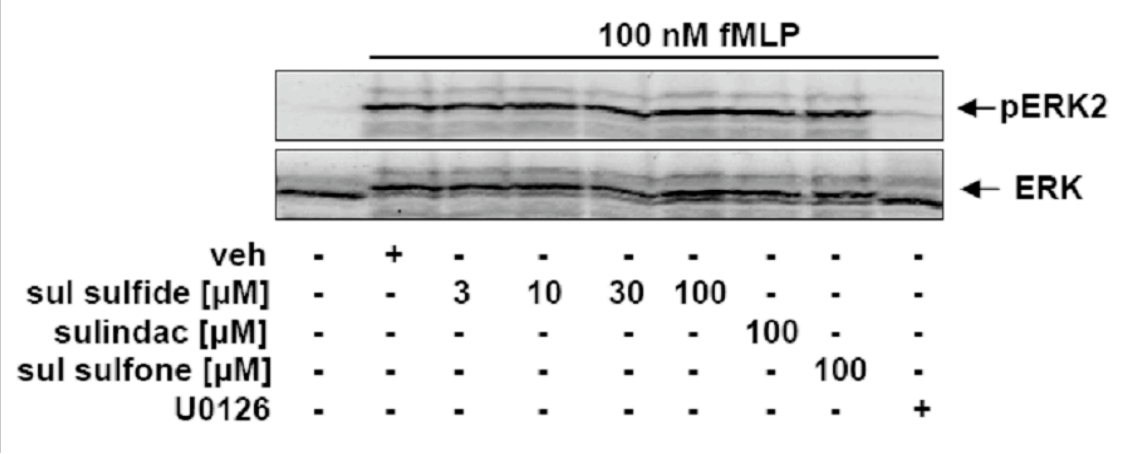


Fig. 31: *Effect of Ssi on ERK-phosphorylation in fMLP-stimulated PMNL*

$1x10^7$ PMNL were incubated with the Ssi and its metabolites concentrations as indicated. U0126 was used as control inhibitor. Samples were stimulated with fMLP (100 nm) to induce 5-LO product synthesis. After the reaction, cells were mixed with 5xLB and boiled for cell lysis and preparation for SDS-PAGE. Western blot analysis followed. A representative of three independent experiments was shown.

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4.2.5 Recovery of Ssi-suppressed 5-lipoxygenase activity by the addition of microsomes to human recombinant 5-lipoxygenase enzyme

A loss of Ssi's potency on 5-LO product formation in cell homogenates and S100 of PMNL was observed in former experiments. As investigations concerning the underlying mechanism revealed that Ssi may interfere directly with the 5-LO enzyme, the following studies were designed to mimic the situation present in cell homogenates by addition of microsomes from PMNL to the purified 5-LO enzyme [328]. Microsomes, for example, are present in broken cell preparations but disappear by centrifugation in the 100.000xg supernatant. Recombinant, human 5-LO was expressed in *E.coli* and purified by ATP-affinity chromatography. Microsomes from PMNL were prepared as described in *Materials and Methods* and preincubated for 15 min on ice with recombinant enzyme before test compounds were added. 5-LO enzyme activity was started with the addition of Ca^{2+} and AA. Ssi alone caused ~ 80 % enzyme inhi-

bition. Samples coincubated with 100 µg microsomes and Ssi led to only ~ 50 % inhibition of 5-LO product formation.The preincubation of 300 µg microsomes with 5-LO enzyme resulted in a completely restored 5-LO catalytic activity and blocked 5-LO inhibition by Ssi.

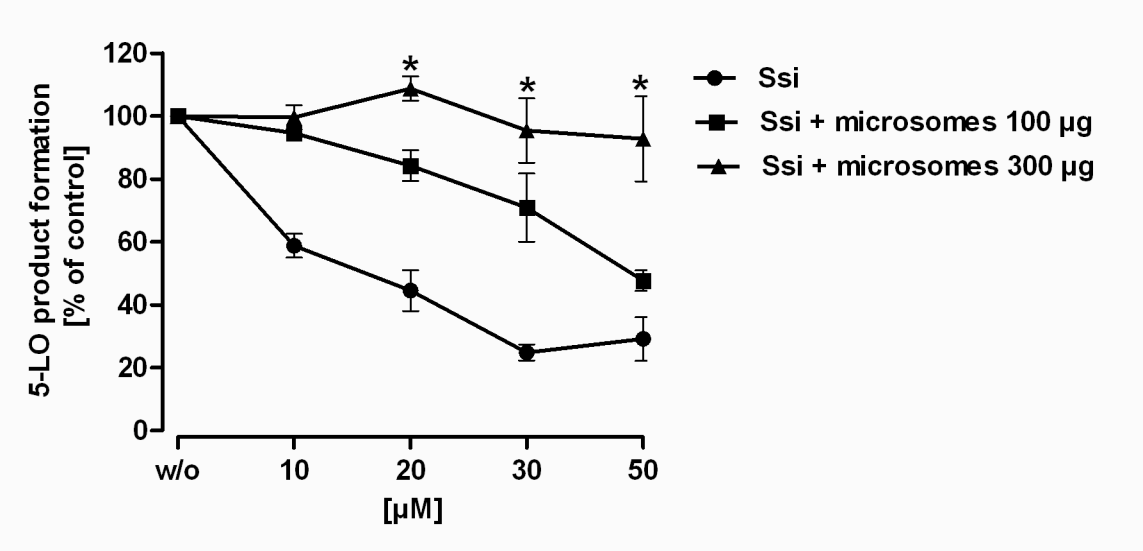

Fig. 32: *Influence of exogenously added microsomes on inhibition of recombinant 5-LO in the presence of Ssi*

*Different amounts of microsomes from PMNL (total protein 100 or 300µg) were preincubated 15 min at 4°C with recombinant 5-LO and then Ssi was added at the indicated concentrations for another 15 min. After starting the reaction by the addition of Ca^{2+} (2 mM) and AA (20 µM), 5-LO metabolites were subjected to HPLC analysis. n=3. * p ≤ 0.05. Values are expressed as mean + SEM.*

To investigate, whether the impaired inhibitory potency induced by microsomes is unique to Ssi, two sets of controls were designed. First, as negative control the well-recognized iron-ligand inhibitor BWA4C was utilized interfering with the non-heme iron located in the center of the catalytic unit of the enzyme. The compound is only slightly affected in its inhibitory potency by the addition of 300 µg/sample microsomes at 1 µM. (Fig. 33).

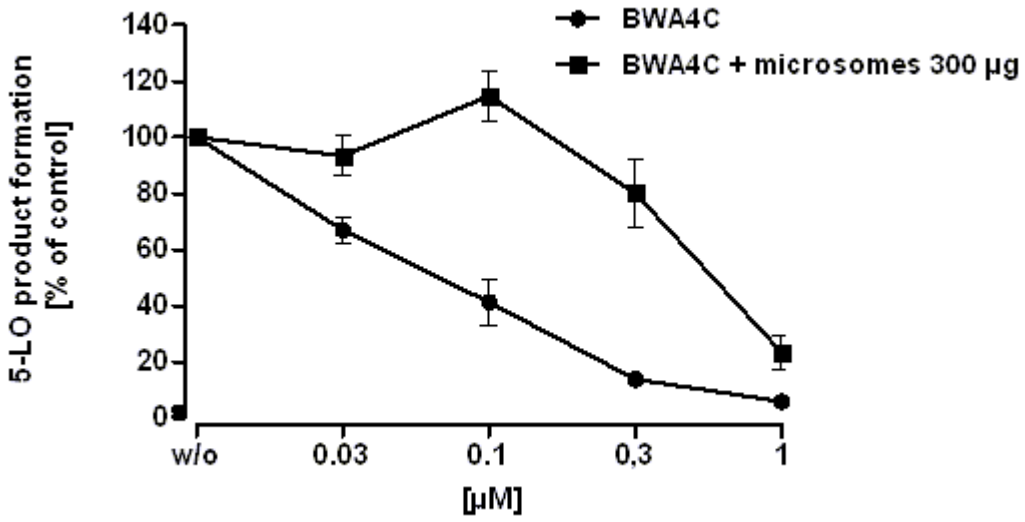

Fig. 33: *Influence of the addition of PMNL-derived microsomes on inhibition of recombinant 5-LO by BWA4C*

Purified 5-LO was investigated for 5-LO product formation by incubation either with BWA4C in different concentrations or by BWA4C and microsomes (300 µg) added to the reaction mix. Samples were stimulated by the addition of Ca^{2+} 2 mM and AA 20 µM. Examination of metabolites was carried out by HPLC analysis. n=3. Values are expressed as mean + SEM.

Secondly, hyperforin, the major component of St.John's wort (*Hypericum perforatum*) and a potent inhibitor of 5-LO which acts by interfering with the C2-like-domain of 5-LO, was chosen for positive control (refer to Fig.11, section 1.2.9) [309]. Hyperforin clearly reduced 5-LO activity down to ~ 20 % product formation at 10 µM. Hence, preincubation with microsomal fractions (300 µg) impaired these effects notably and restored complete 5-LO enzyme activity.

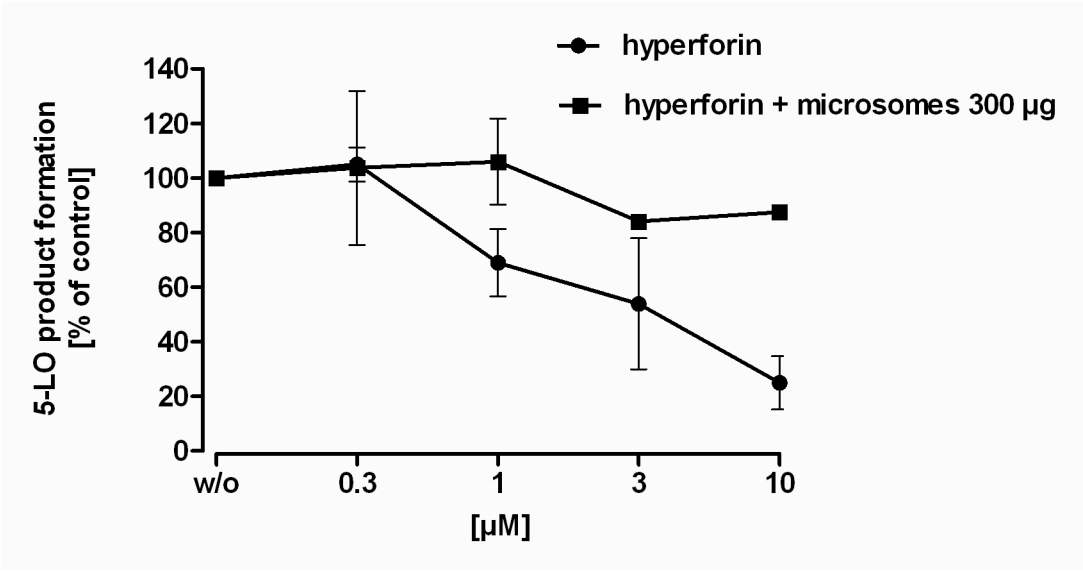

Fig. 34: *Influence of the addition of PMNL-derived microsomes on inhibition of recombinant 5-LO by hyperforin*

Purified 5-LO was investigated for 5-LO product formation by incubation either with hyperforin in different concentrations or by hyperforin and microsomes (300 µg) added to the reaction mix. Samples were stimulated by the addition of Ca^{2+} (2 mM) and AA (20 µM). Determination of the formed 5-LO metabolites was carried out by HPLC analysis. n=3. Values are expressed as mean + SEM.

4.2.6 Influence of phospholipids on 5-lipoxygenase inhibition by Ssi

From the data shown on the previous section where microsomes impaired the inhibitory efficacy of Ssi on 5-LO, extended investigations were carried out to obtain more detailed information about a possible binding of Ssi to the regulatory domain C2-like of 5-LO. The following experiment analyzed the capacity of PC to counteract the inhibition of 5-LO activity induced by Ssi. For testing this, purified enzyme was incubated with two different types of phospholipids such as PC and PE at concentration of 100 µg/ml in PBS/EDTA buffer and Ssi was directly added to the reaction mixture. Stimulated samples (Ca^{2+}, 2 mM) were supplemented with substrate (AA 20 µM) and the formed 5-LO metabolites were determined by HPLC analysis. In Fig. 35 Ssi-suppressed 5-LO product formation was visible in absence of phospholipids. Only in the presence of PC and Ssi enhanced 5-LO product formation was detected compared to the data obtained with PE and Ssi. This is reflected in the IC_{50} values that range from ~ 30 µM (100 µg/ml PE +Ssi) to >100 µM (100 µg/ml PC +Ssi).

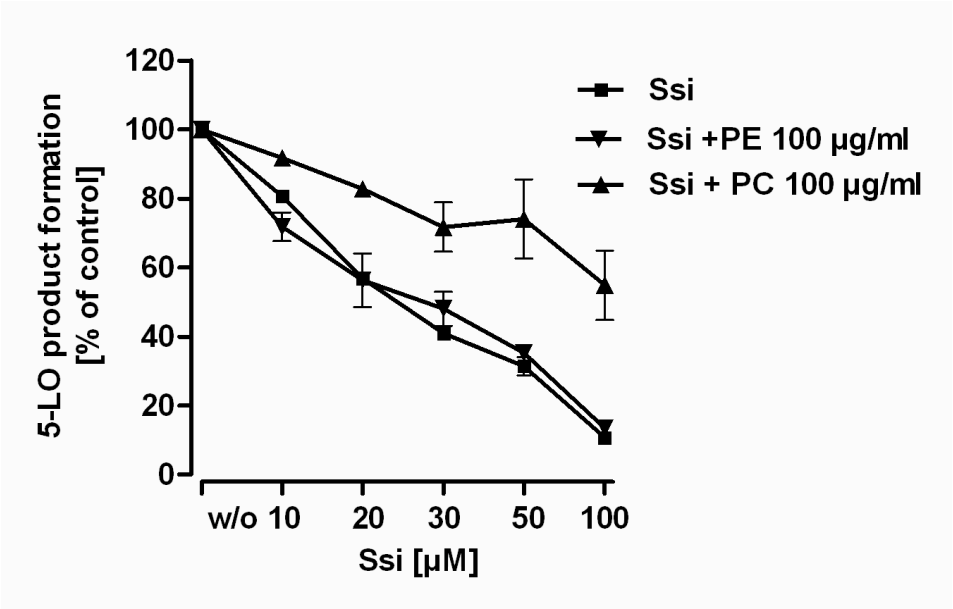

Fig. 35: *Influence of PC and PE on Ssi-mediated 5-LO inhibition*

PE (100 µg/ml) and PC (100 µg/ml) were incubated together with increasing concentrations of Ssi and puified, recombinant enzyme. Stimulation with Ca^{2+} (2 mM) and AA (20 µM) initiated 5-LO product formation followed by detection via HPLC. Samples incubated with Ssi were calculated in relation to the control with only 10 µg/ml PC, respectively. n=3. Values are expressed as mean + SEM.

Next, dose response characteristics of PC were determined. In line with Fig. 35, purified 5-LO enzyme was incubated with PC in increasing concentrations. In this membrane-mimicking model an abolition of suppression of 5-LO product formation by 100 µM Ssi is observed by titrating increasing concentrations of PC to the reaction mixture (Fig. 36). Ssi suppressed the 5-LO activity level to ~ 10 % remaining enzyme activitiy which did not differ from samples incubated with 1 µg/ml PC. But at a concentration of 3 µg/ml PC 5-LO enzyme activity started to be restored from ~ 30 % - ~ 60 %. Differences between samples with Ssi treatment and samples treated with Ssi+100 µg/ml PCl were significant (Fig. 36).

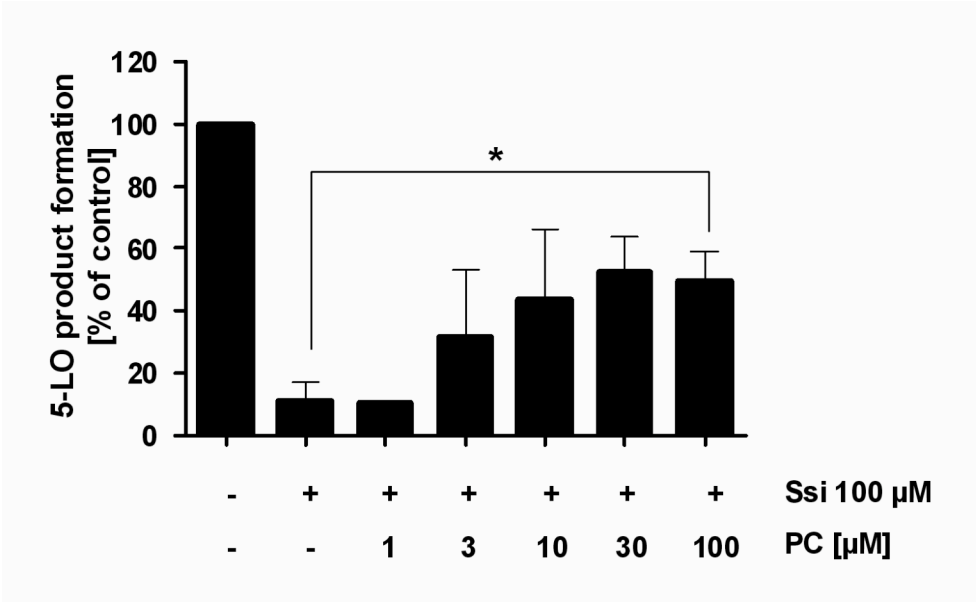

Fig. 36: *Influence of increasing amounts of PC on Ssi-mediated 5-LO inhibition*

*Samples containing 5-LO enzyme in buffer were analyzed regarding 5-LO metabolite production after incubation with Ssi (100 µM) or Ssi (100µM) and increasing concentrations of PC [µg/ml] as indicated. The control was determined in ratio to product formation in samples were enzyme was incubated with only PC in the concentrations indicated. * p ≤ 0.05. Values are expressed as mean + SEM.*

In order to confirm the monitored effect of PC and to exclude phospholipid mediated unspecific effects on Ssi, two further phospholipids, without affinity to the C2-like-domain of 5-LO enzyme [214] were tested under the same conditions. A concentration of 100 µg/ml PC counteract the inhibitory effect of Ssi on 5-LO and based on that, concentrations of PE and PS were adapted. As control, 5-LO activity assays were conducted with only Ssi or with lipids alone. Samples incubated with Ssi+ PC, PE or PS (100 µg/ml), respectively were analyzed for 5-LO product formation. Suppression of 5-LO by Ssi was validated by the incubation with 100 µM Ssi and only ~ 10 % 5-LO activity remained. In contrast, PC increased the 5-LO product formation to about 60 % of control which is in line with the previous findings (Fig. 36). This was not observed for the samples incubated with PE or PS in the presence of Ssi where 5-LO activity remained at ~ 20 % and behaved like the Ssi control (Fig. 37).

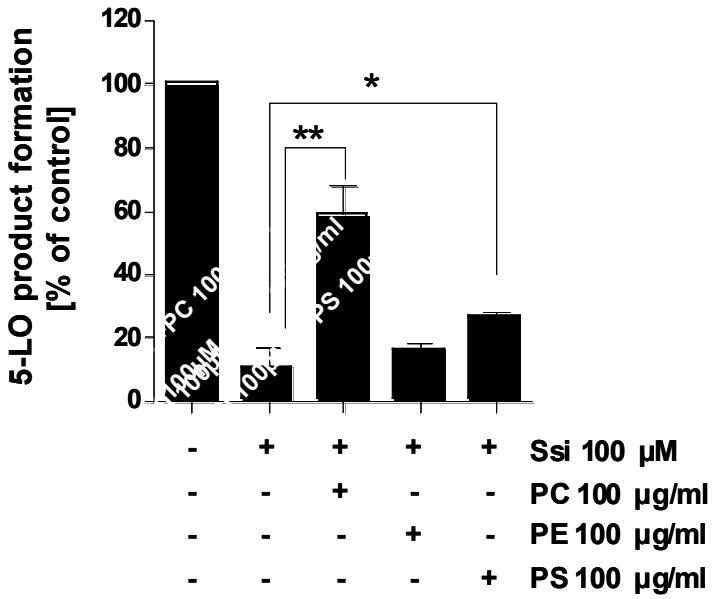

Fig. 37: *Comparison of the influence of different lipids on Ssi-mediated 5-LO suppression*

*Recombinant 5-LO was incubated with Ssi at 100 µM alone, with different phospholipids alone (PC, PE, PS, c=100 µg/ml) or with the combination of Ssi and phospholipids. 10 min after starting the reaction by the addition of 2 mM Ca^{2+} and supplementation of 20 µM AA, metabolites were detected by HPLC.n=4. ** p=0.01; * p≤ 0.05. Values are expressed as mean + SEM.*

To ensure that the determined effects were not underlying any unspecific mechanisms of interactions between Ssi and the lipids, the following experiments addressed whether other 5-LO inhibitors with well-recognized binding modes were influenced in their inhibition efficacy in presence of PC. BWAC, also used in former experiments, shows the profile of an iron-ligand-inhibitor of 5-LO interfering with the non-heme-iron in the enzyme's catalytical domain [282]. Therefore, experiments were carried out by incubating 5-LO enzyme with BWAC 1 µM and BWA4C 1 µM in addition to 100 µg/ml PC (Fig. 38). Supplementary, Ssi was again tested for 5-LO inhibition alone or in addition with PC. As expected, almost completely abolished enzyme activity was obtained by incubation of Ssi and BWA4C alone with purified 5-LO. But the reversibility of the 5-LO inhibition mediated by PC only occurred in samples incubated with Ssi and PC, not with BWA4C and PC.

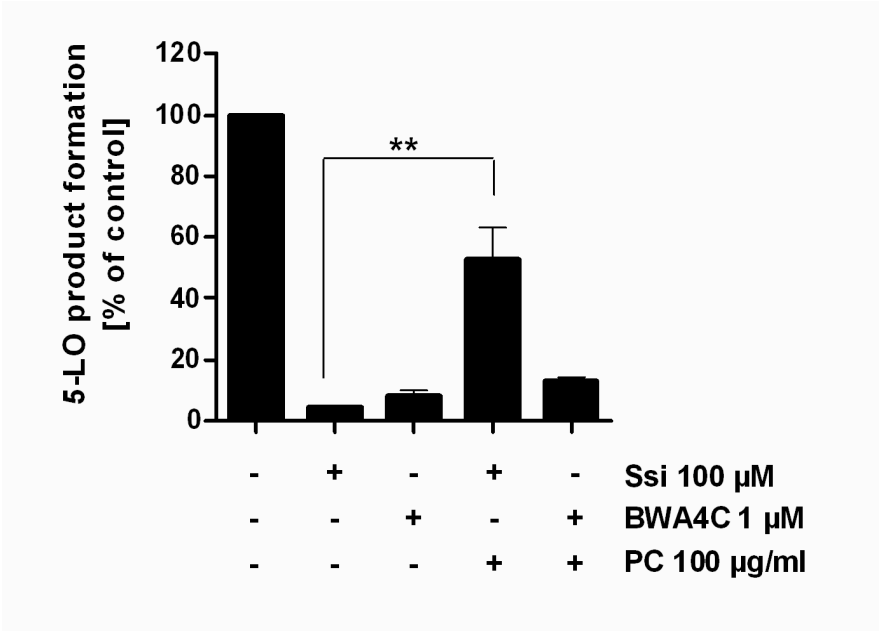

Fig. 38: *Influence of PC on 5-LO product formation in the presence of Ssi and BWA4C*

*Samples were incubated with compounds and with or without PC with concentrations as indicated. 5-LO product synthesis was started with Ca^{2+} (2 mM) and ensured by supplementation of substrate AA (20 µM). Afterwards, products were detected by UV-absortion by HPLC. n=3. ** p ≤ 0.01. Values are expressed as mean + SEM.*

4.2.7 Effect of Ssi on recombinant cyclooxigenase-2 activity in presence of lipid vesicles

It is known from literature that phospholipid molecules are assumed to ressemble biological membranes by the formation of liposome vesicles and to emulate interactions with drugs, they can function as artificial membranes [329]. The following section deals with the question whether the observed effect of PC is due to a competition with Ssi for the same molecular target or due to the fact that formed liposome vesicles of PC in the reaction mix circumvent the possibility of Ssi to reach 5-LO while trapped by liposomes. In order to determine a potential trapping of Ssi by PC vesicles, a COX-2 activity assay was performed as suppression of COX-activity displays Ssi's so far known mode of action. Human recombinant COX-2 was incubated in a sample set only with Ssi in several concentrations and together with PC 100 µg/ml in a second sample set. The formed product was assessed with an enzyme-linked immuno assay (ELISA). As internal assay control, the selective COX-2 inhibitor rofecoxib was used at 1 µM concentration, also in presence or absence of PC

100µg/ml. Rofecoxib suppressed COX-2 activity very potently (only 20% residual COX-2 activity left) in comparison to Ssi (~ 60 % residual COX-2 activity). However, the presence of PC did not change the COX-2 inhibition profile of Ssi in comparison to the exclusively Ssi-treated control sample set (Fig. 39).

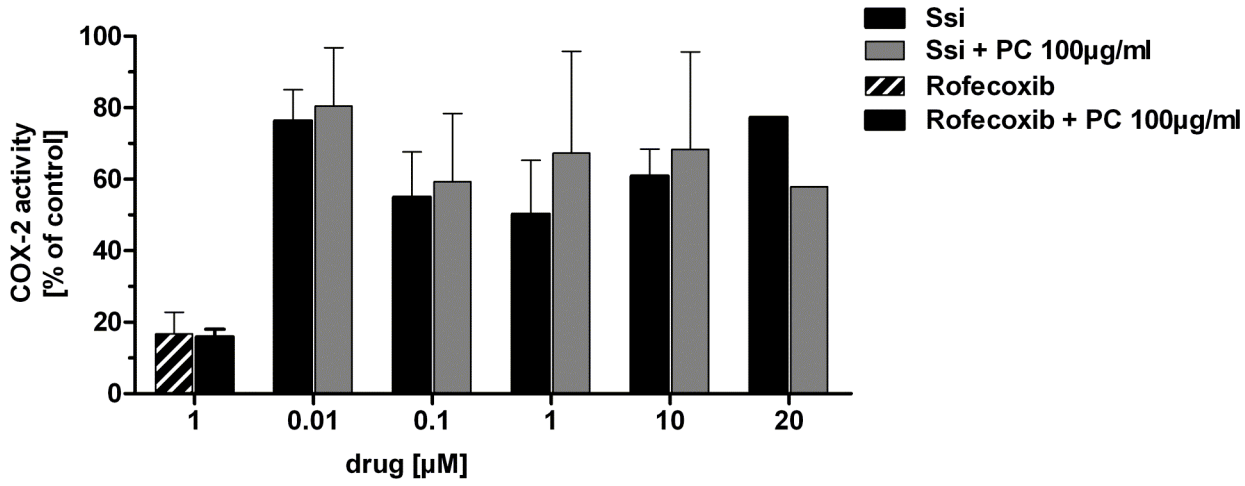

Fig. 39: *Effect of Ssi on recombinant COX-2 activity in presence of lipid vesicles*

COX-2 enzyme was incubated with Ssi in concentrations indicated and in another example PC (c=100 µg/ml) was added to increasing Ssi concentrations. COX-2 activity was measured by ELISA. n=2.

4.2.8 Identification of the target structure of Ssi within the regulatory C2-like domain of 5-lipoxygenase

To confirm the data so far obtained suggesting an interaction of Ssi with a lipid binding site, further studies were undertaken to elucidate whether Ssi shows a direct binding to the regulatory domain of the 5-LO enzyme. It has been demonstrated that lipid binding to the C2-like-domain, resulting in augmented 5-LO activity, is mediated by three tryptophan residues (W12, W75, W102) in the regulatory domain [210]. Therefore the existing mutant of the 5-LO enzyme (5-LO-3W), where the three tryptophane residues were changed to alanin by site-directed-mutagenesis PCR [218], was incubated with Ssi in different concentrations. Both proteins (5-LO-wt and 5-LO-3W) were stimulated with $CaCl_2$ (2 mM) and AA (20 µM) after incubation with Ssi up to 50 µM (in relation to the concentrations achieved in humans after oral doses) for 15 min at 4°C and were analyzed for their amount of formed 5-LO metabolites by HPLC. 5-LO activity only moderately differed up to concentrations of 20 µM Ssi if 5-

LO-wt is compared to the 5-LO-3W. Higher concentrations of Ssi (30 µM) resulted in 50 % 5-LO-wt enzyme activity while 5-LO-3W showed ~ 80 % remaining enzyme activity. 5-LO-wt activity is suppressed to almost zero level at 50 µM Ssi while 5-LO-3W remained at ~ 70 % remaining activity (Fig. 40).

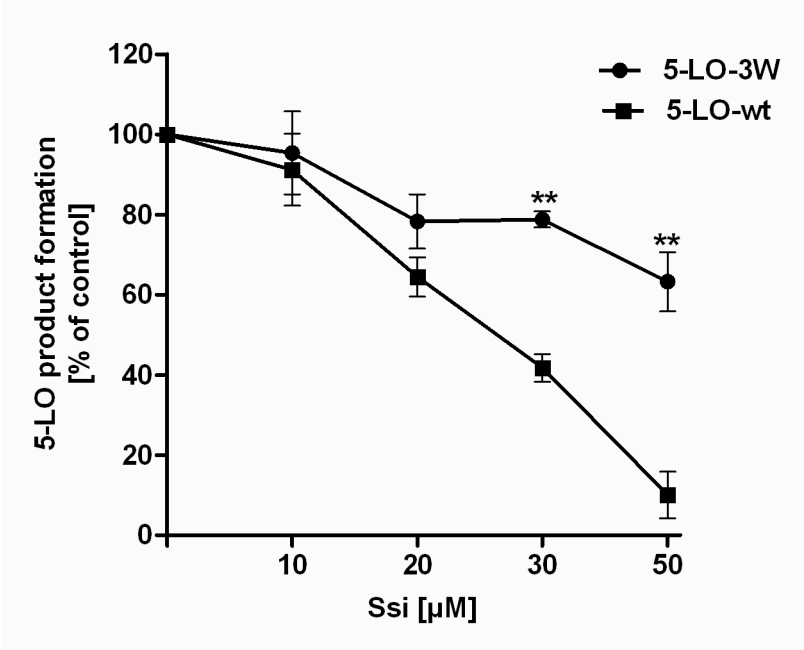

Fig. 40: *Inhibitory effect of Ssi on 5-LO-wt and -5-LO-3W product formation*

*Both proteins, expressed in E.coli and purified by ATP-affinity-chromatography, were incubated with the same concentrations of Ssi ranging from 10-50 µM and analyzed for 5-LO product formation after addition of 2 mM Ca^{2+} and 20 µM AA.n=4. ** $p \leq 0.003$. Values are expressed as mean + SEM.*

Again, it has to be clarified whether the lack of inhibition of Ssi with the 5-LO-3W mutant is an unspecific side effect or uniquely for the mode of action of Ssi on 5-LO-wt enzyme. In order to specify the effect, another inhibitor class (BWA4C) was used. Compared to 5-LO-3W, 5-LO-wt enzyme activity is almost completely repressed by Ssi and less than 20 % remaining activity is determined in the 5-LO activity assay while the 3W-mutant is again not affected at the highest suppressive concentration of Ssi used (50 µM). BWA4C potently inhibited 5-LO-wt enzyme activity but led to a somewhat higher residual 5-LO product formation of 5-LO-3W (~ 30 %) as shown in Fig. 41.

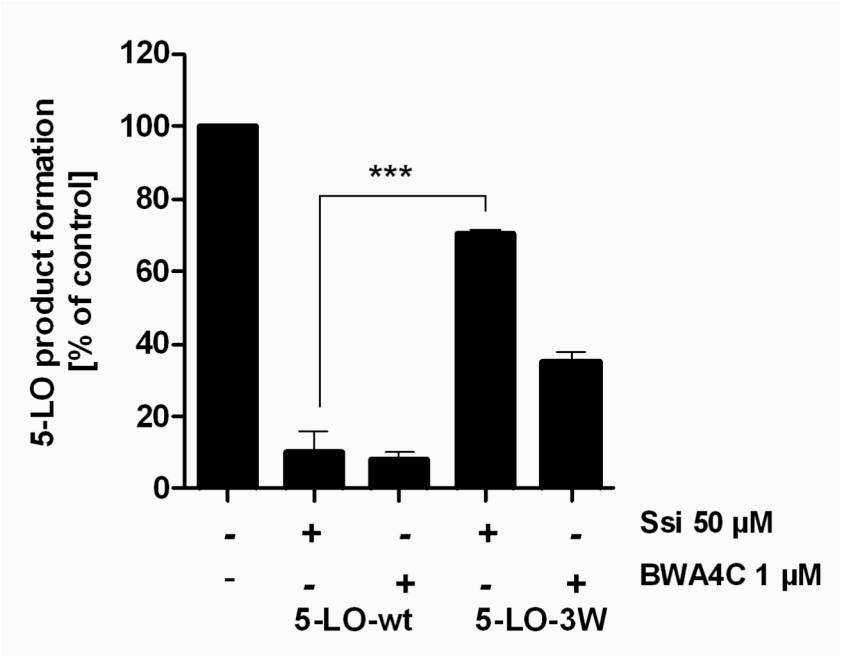

Fig. 41: *5-LO inhibition by Ssi or BWA4C on 5-LO-wt and 5-LO-3W*

*5-LO product formation was analyzed in both purified, recombinant proteins. Ssi was used at a high 5-LO-wt suppressive concentration of 50 µM and BWA4C at well-known 5-LO inhibiting concentration of 1 µM. HPLC analysis was performed after Ca^{2+}-induced stimulation and supply of enough endogenous substrate AA (20 µM).n=3. *** $p \leq 0.005$. Values are expressed as mean + SEM.*

To further characterize the inhibitory efficacy of BWA4C on 5-LO-3W and to possibly evaluate concentration-dependent differences, BWA4C was tested for 5-LO inhibition in both protein-mixtures and dose-concentration curves were recorded (Fig. 42). The proteins were incubated with increasing concentrations of BWA4C ranging from 0.03 µM to 1 µM. In Fig. 42, a slight difference in the inhibitory potency between the 5-LO-wt and the 5-LO-3W could be observed, but 1 µM BWA4C almost completely suppressed the activity of both proteins.

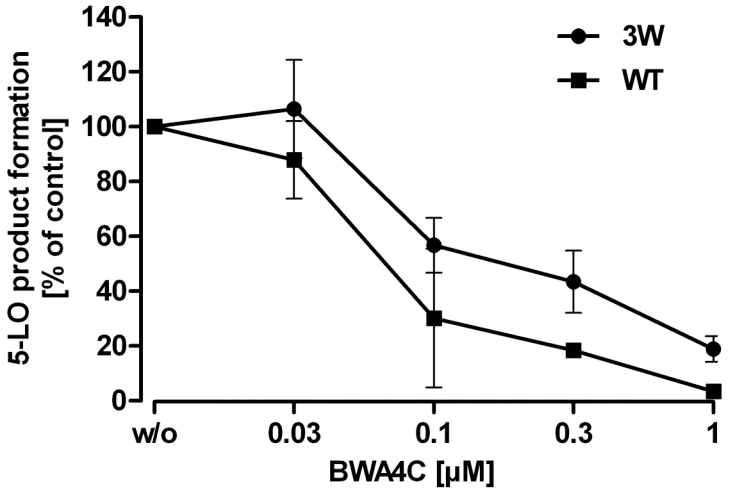

Fig. 42: *Dose-response-curves of the 5-LO inhibitor BWA4C for 5-LO-wt protein and mutated 5-LO-3W*

5-LO-wt and 5-LO-3W enzymes were incubated with increasing BWA4C concentrations for 15 min at 4°C and then stimulated with Ca^{2+}/AA (2 mM /20 µM) for 10 min at 37°C.Then formed metabolites were determined by HPLC. n=3. Values are expressed as mean + SEM.

Finally, studies were undertaken to elucidate the influence of PC and ist effect on the activity of wildtype 5-LO and mutated 5-LO-3W. 5-LO-wt enzyme showed almost no activity in presence of Ssi (50 µM) alone in contrast to the samples were Ssi and PC were present. Here 5-LO activity reached ~ 400 % of relative product formation over control. Regarding the mutated 5-LO protein, enzyme activity was still not impacted by Ssi 50 µM and even the addition of PC 30 µg/ml did not show any stimulation of 5-LO-3W enzyme activity to levels obtained for the 5-LO-wt (Fig. 43).

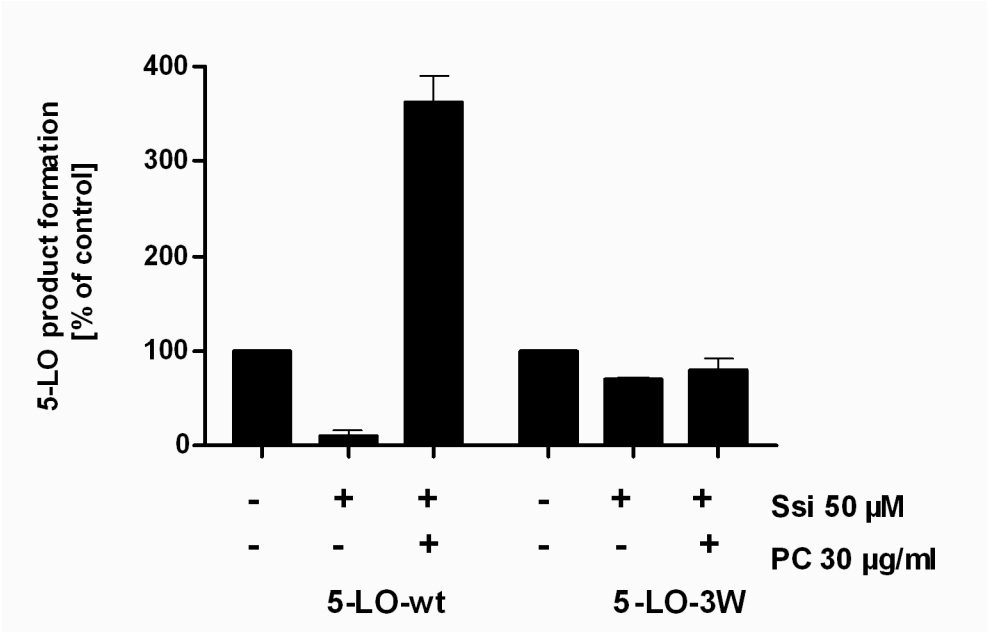

Fig. 43: *Effect of Ssi and PC on enzyme activity of both 5-LO proteins*

Proteins were analyzed for activity after incubation with Ssi alone (50 µM) or in combination with PC (30 µg/ml) and stimulated with 2 mM Ca^{2+} and 20 µM AA. Formed 5-LO metabolites were determined by HPLC analysis. n=3. Values are expressed as mean + SEM.

4.3 Regulation of 5-lipoxygenase expression by Ssi

Although the chemopreventive and antitumorigenic activities of NSAIDs against several types of cancer are well established, the molecular mechanisms responsible for these properties still remain to be elucidated [62]. Inhibition of COX-1 and COX-2 leading to impaired PG biosynthesis represents the major anti-carcinogenic mechanism of action of NSAIDs. Considerable evidence accounts for a role of partially COX independent actions in the drugs chemopreventive activity [61]. Ssi possesses a distinct chemopreventive activity with molecular mechanisms only partly understood and could recently being identified as a potent inhibitor of 5-LO [328]. For the 5-LO enzyme obviously playing a crucial role in cancer progression as reported in literature, it seemed reasonable to elucidate whether the drug may also modulate 5-LO gene expression. Though it is known that the promotor region of 5-LO is regulated by Sp1 on transcriptional level, the question raised whether Sp1 as a transcriptional regulation factor is possibly involved [175]. MM6-cells were chosen for the following expression analysis as these monocytes possess an inducible 5-LO after treatment with a combination of calcitriol and the cytokine TGF-ß [315].

4.3.1 Analysis of 5-lipoxygenase mRNA levels in Ssi-treated MM6-cells

First, to get an overview about a possible inhibition of 5-LO expression by Ssi treatment, MM6-cells were examined for 5-LO gene expression after differentiation with calcitriol/TGF-ß and incubation with Ssi on mRNA level via qPCR. Cells were differentiated for 72 hrs at 37°C in a 6 % CO_2 atmosphere and after that Ssi was added in six different concentrations. As nothing was known so far about Ssi's capacity to influence 5-LO gene expression, concentrations were chosen likewise on the basis of the published data refering to the COX gene regulation [61, 62]. Various incubation times with the drug were tested and the most prominent effects were observed after an incubation time of 72 hrs with Ssi. Parallel, the same experiment was carried out with undifferentiated MM6 cells to monitor whether these effects are differentiation related. After harvesting and washing the cells, RNA was isolated using the total RNA Mini Kit according to the manufacturer's protocol. RNA concentrations were determined and 2 µg RNA were transcribed into cDNA with the I-script® cDNA synthesis kit. Amplification over the 5-LO proximal promotor region (-286 to +78 base pairs relative to transcription initiation site) was performed for 32 cycles. The housekeeping gene ß-actin was used as control template and results are presented as 5-LO copy numbers per 1000 copies of ß-actin. Fig. 44 shows that treatment with Ssi provoked a decrease in 5-LO mRNA levels in a concentration-dependent manner up to 30 µM of Ssi in comparison to the untreated control. In undifferentiated cells, a drecrease in 5-LO mRNA was also visible, but to a somewhat lower extent than in differentiated cells. As expected, only a marginal 5-LO mRNA level was achieved in all samples.

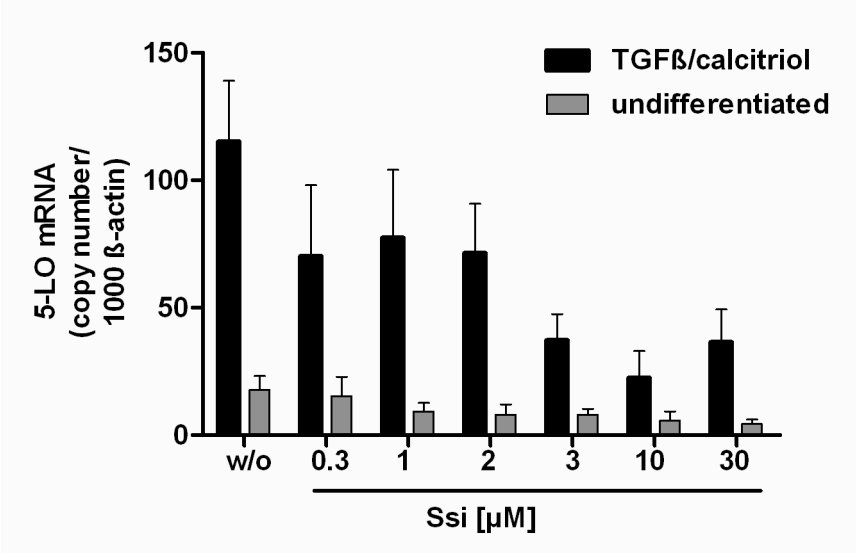

Fig. 44: *Quantification of 5-LO mRNA levels in MM6-cells after Ssi treatment for 72 hrs*

$0.6x10^6$ differentiated (calcitriol 50 nM / TGF-ß 1 ng/ml; 72 hrs) and undifferentiated cells were incubated with Ssi in concentrations as indicated for 72hrs. Realtime PCR was performed for 32 cycles after cell harvest, mRNA extraction and transcription into cDNA. 5-LO copy numbers were normalized to 1000 ß-actin copies. n=3. Values are expressed as mean + SEM.

4.3.2 Analysis of Sp1 mRNA levels in Ssi-treated MM6-cells

As there exists knowledge in former published data that the transcriptional factor Sp1 is involved in activation of the 5-LO promoter region by a specific binding site, the so-called GC4-box, the next experiments dealed with the analysis of Sp1 mRNA levels in MM6-cells treated with Ssi for 72 hrs. The same experiments as described in section 4.4.1 were carried out to analyse the amounts of Sp1 mRNA in differentiated MM6-cells versus undifferentiated cells. In differentiated MM6-cells, Sp1 levels were visibly decreased after the addition of increasing concentrations of Ssi. At 10 µM and of 30 µM Ssi, the effect is most pronounced leading to 50 % loss of Sp1 mRNA amount compared to DMSO control. However, MM6-cells not treated with calcitriol/ TGF-ß were not able to show significant differences in Sp1 mRNA amounts.

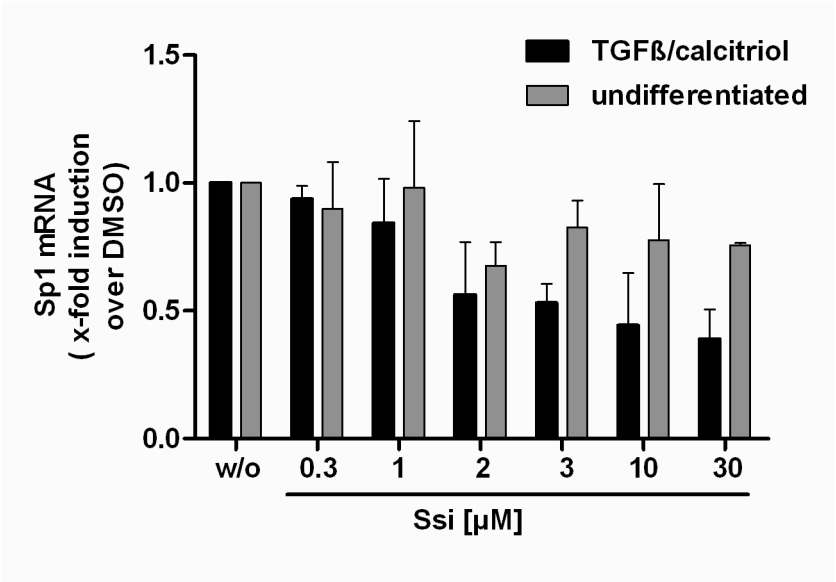

Fig. 45: *Quantification of Sp1 mRNA levels in MM6-cells after Ssi treatment for 72 hrs*

$0.6x10^6$ differentiated (calcitriol 50 nM / TGF-ß 1ng/ml; 72 hrs) and undifferentiated cells were incubated with Ssi in concentrations as indicated for additional 72hrs. mRNA levels were assessed by qPCR using specific primers for Sp1. Results are expressed as x-fold induction over DMSO control.

4.3.3 Cotransfection of Sp1 and 5-LO-pN10 in reporter gene assay in HeLa cells

So far, investigations concerning the underlying mechanism of 5-LO regulation by Ssi on transcriptional level revealed an interaction of Sp1 and Ssi. The following experiment was undertaken to understand if Sp1 is directly involved in Ssi-mediated downregulation of 5-LO. In order to confirm this, a reportergene assay model with HeLa cells was chosen as an appropriate test system. After 24 hrs of cotransfection of a 5-LO promotor plasmid (pN10) and a pSG5-Sp1 expression plasmid, media was changed and cells were incubated with three different concentrations of Ssi. After another 16 hrs, reporter gene activity was measured with to the Dual Glow® system. Values are calculated as reduction of pN10 activity over backbone pSG5. As shown in Fig.46, Ssi treatment counteracted the Sp1-mediated 5-LO promotor activation in a concentration-dependent manner. Due to maybe reduced capacity of transfected Sp1 to increase 5-LO promoter activity at 50 ng, a second concentration of 100 ng was used. However, no significant differences were detectable excluding the samples treated with 3 µM. 10 µM and 30 µM of Ssi led to a clear counteraction of 5-LO promoter activation triggered by Sp1 almost comparable to DMSO controls.

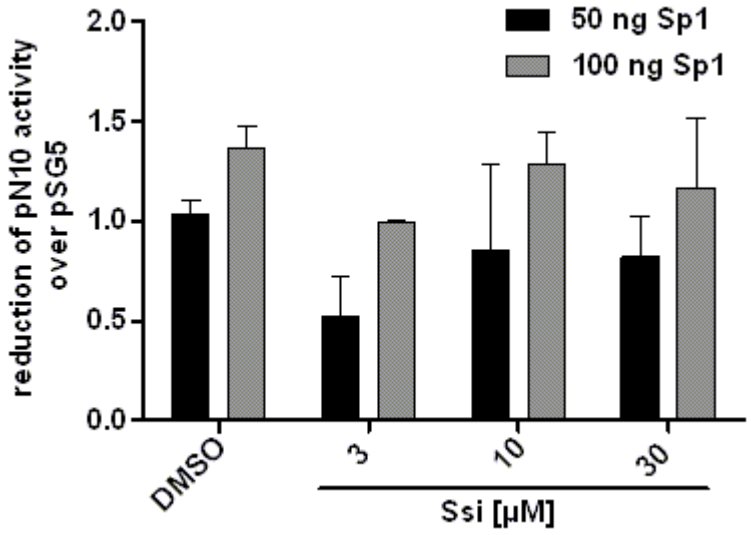

Fig. 46: *Reporter gene analysis of cotransfection of 5-LO pN10 and Sp1 pSG5 in HeLa cells*

$4x10^4$ HeLa cells were seeded out 24 hrs prior to transfection. Cotransfection takes places for 24 hrs and then media was changed and samples were incubated with Ssi in the indicated. After 16 hrs luciferase activity was measured and values are calculated by standardization of reportergene luciferase to renilla-luciferase.n=3.

4.3.4 ChIP analysis of Sp1 recruitment to the 5-lipoxygenase promoter in Ssi-treated HeLa cells

Furthermore, another experiment dealing with the underlying mechanisms of Ssi-mediated downregulation of 5-LO mRNA, is now described. Results of the precedent section suggest that Sp1 may play a role in the 5-LO downregulation observed in MM6-cells by treatment with Ssi. Therefore, ChIP assays should give more detailed information about a possible interplay between Ssi and Sp1. In brief, cells were grown, either differentiated or undifferentiated, treated with Ssi for 72 hrs and 16 hrs prior to harvest, MS-275, a selective histone-1,3-deacetylase-inhibitor (HDAC inhibitor) was added (10 µM) to circumvent possible involvement of HDACs. After cell harvest ($1x10^7$), cells were fixed with 1% formaldehyde-solution and processed for further precipitation against Sp1 antibody and normal rabbit IgG. A sample using no antibody served as internal assay control. DNA-templates were then amplified by RT-PCR. In line with results from qPCR and Western blot analysis (refer to section 4.4.1-4.4.2), the same regulation pattern of Sp1 resulted after treatment of cells with Ssi in the ChIP assay. Reduced Sp1 binding at 3 µM Ssi occured in differentiated MM6

cells which is completely abolished at 30 µM Ssi. IgG was used as control antibody. Input was determined via specific primers supplied from the manufacturer. It can clearly be seen that Sp1 is downregulated in MM6-cells whilst treated with Ssi 3 µM and 30 µM.

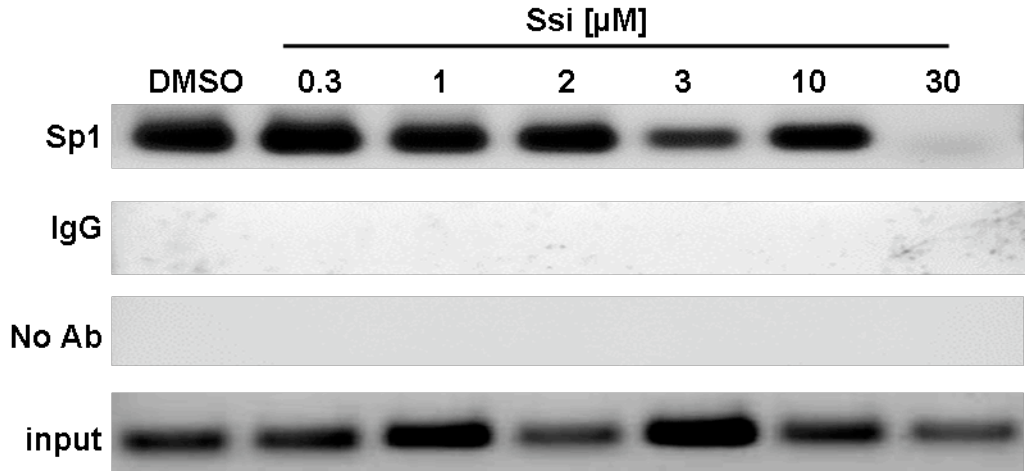

Fig. 47: *Sp1 ChIP assay of differentiated MM6-cells treated with Ssi for 72hrs*

1x10^7 MM6 cells differentiated with calcitriol/TGF-ß for 72 hrs were incubated with Ssi for additional 72 hrs and then harvested. ChIP assay was performed against Sp1, rabbit IgG and then templates were amplified by RT-PCR. No Ab and input samples were used as internal assay controls.n=1.

The same experimental setup was used for undifferentiated MM6 cells to see if there occurs as well Sp1 downregulation. Shown in Fig. 48, a loss of Sp1 is visible at 30 µM Ssi as well compared to the ChIP analysis in differentiated MM6 cells. But if intensities of 3 µM Ssi treatment are compared, clearly reduced Sp1 binding occurred in differentiated MM6-cells whereas undifferentiated cells showed no change in the intensitiy of Sp1 lane.

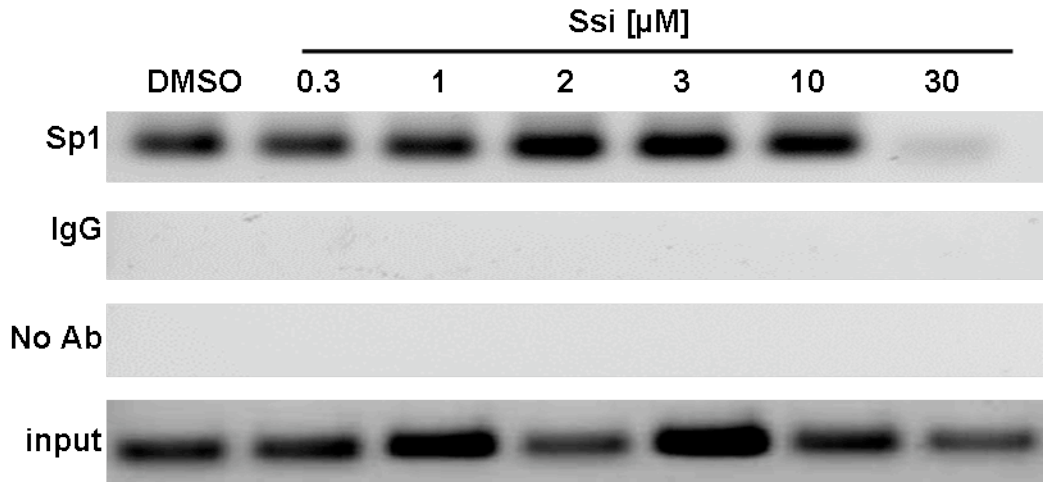

Fig. 48: *Sp1 ChIP assay of undifferentiated MM6-cells treated with Ssi for 72hrs*

ChIP results in MM6 cells (1x10^7) incubated with Ssi for 72hrs and then harvested. ChIP assay was performed against Sp1, rabbit IgG and then templates were amplified by RT-PCR. No Ab and input samples were used as internal assay control.

4.4 5-lipoxygenase - a potential target for nitrosylated NSAIDs

In the literature, an increasing body of evidence refers to NO-NSAIDs as compounds demonstrating superior effects against a variety of cancer types in cell cultures systems and animal models.They promise contribution to the control of cancer but the molecular mechanisms are not clear so far. Furthermore, NO-releasing NSAIDs seem to be able to protect against NSAID-induced gastric damage in rats which could be due to the effects of NO [330]. NO-NSAIDs possess a conventional NSAID moiety linked, often via a spacer molecule to an NO-releasing moiety [66]. To elucidate whether NO-NSAIDs possibly target 5-LO the following section deals with the characterization of well-known NSAIDs and their corresponding nitrosylated forms. In this work, sulindac, naproxen and acetylsalicylic acid (ASA) and their NO-counterparts were chosen as test compounds.

4.4.1 Effect of NO-sulindac and NO-naproxen on 5-lipoxygenase activity in intact PMNL

To get any knowledge about a possible difference between the conventional NSAID and the nitrosylated from, intact PMNL were incubated with both, NO-NSAIDs as well as the non-nitrosylated drugs. In order to mimic inflammatory conditions, intact cells were stimulated with the strong chemical stimulus A23187 (2.5 µM). Sulindac, at concentrations of 10 and 30 µM, did not affect 5-LO activity. The nitrosylated form, NO-sulindac suppressed LT biosynthesis at 10 µM to a residual enzyme activity of 20 % and almost zero at higher drug concentrations (30 µM). Beside, another NSAID was tested for potential 5-LO suppression. Naproxen and its nitrosylated derivative showed contrasting effects on LT formation. Naproxen did not influence 5-LO activity but NO-naproxen reduced product formation to ~ 40 % of control. There was no difference in suppression of 5-LO metabolite formation between NO-naproxen used at 50 µM and 100 µM (Fig. 49). (NO-sulindac and NO-naproxen were synthesized by E. Buscato, group of Jun. Prof. E. Proschak, Goethe University Frankfurt)

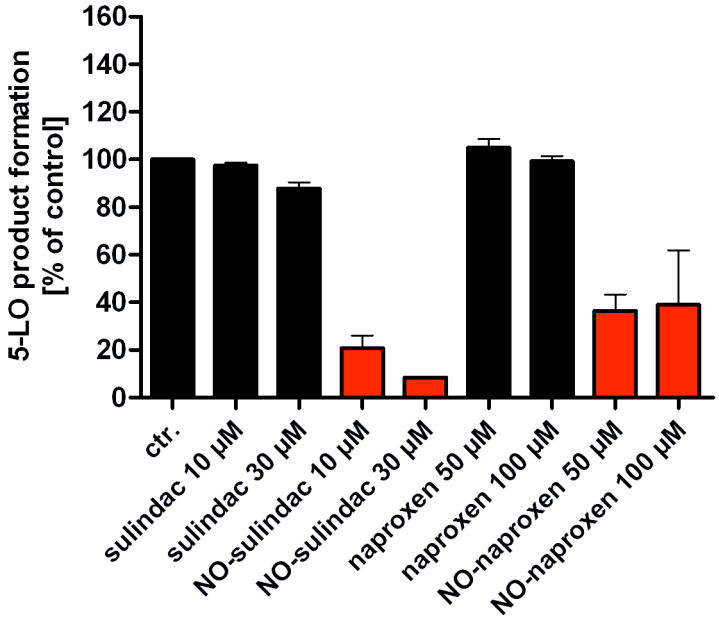

Fig.49: *Influence of NO-sulindac and NO-naproxen on 5-LO activity in intact PMNL*

5x10^6 intact cells (PMNL) were preincubated with NSAIDs respectively NO-derivatives at 4°C with concentrations indicated for 15 min. Samples were then stimulated with 2.5 µM A23187 and supplied with 20 µM AA. Aanalysis of 5-LO products by HPLC was performed.n=3. Values are expressed as mean + SEM.

4.4.2 Influence of NO-sulindac on recombinant 5-LO enzyme

To further elucidate the potency of NO-sulindac, IC_{50} values were determined using human recombinant 5-LO enzyme. Purified 5-LO was incubated with the compounds at the concentrations indicated (refer to Fig. 50) for 15 min on ice. After stimulation of 5-LO using $CaCl_2$ (2 mM) as a stimulus and supplementation of 20 µM AA, 5-LO metabolites were determined by HPLC analysis. The non-nitrosylated sulindac did not influence 5-LO catalysis (~ 100 % enzyme activity). Interestingly, NO-sulindac led to full enzyme inhibition at 10 µM and IC_{50} values can be estimated at ~ 1 µM (Fig. 50).

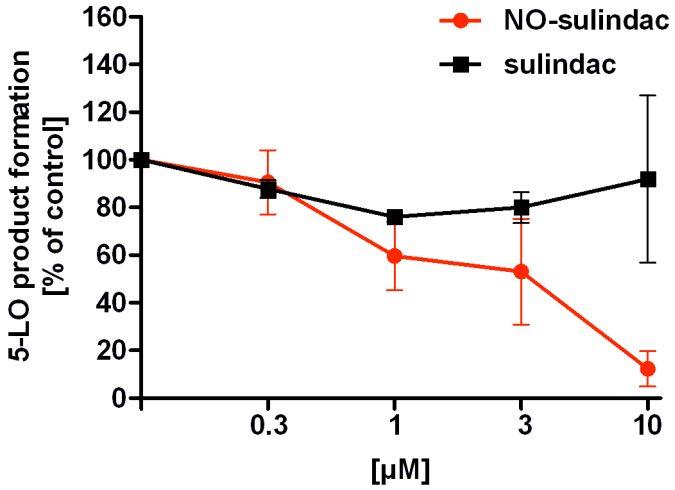

Fig. 50: Influence of sulindac and NO-sulindac *on recombinant 5-LO enzyme activitiy*

5-LO protein was incubated with the concentrations indicated of sulindac and NO-sulindac and analyzed after stimulation with 2 mM $CaCl_2$ and 20 µM AA for 5-LO product formation via HPLC.n=4. Values are expressed as mean + SEM.

4.4.3 Effect of NO-aspirin and aspirin on 5-lipoxygenase inhibition in intact PMNL

In the following studies, the effect of NO-aspirin (NO-ASA) on 5-LO activity was assessed compared to the well-known unselective COX-inhibitor aspirin (ASA). In order to get insights into the mode of action of novel inhibitors, substrate dependency was investigated. Intact PMNL cells were tested for 5-LO actvitiy in the presence of NO-ASA and ASA, respectively without exogenously added AA. Cells were incubated with different concentrations of ASA and NO-ASA. After 15 min 5-LO activity was stimulated by the addition of A23187 (2.5 µM). After determination of 5-LO metabolites by HPLC considerable differences in the obtained concentration-response-curves are received (Fig. 51). Incubations with ASA did not lead to any change in 5-LO product formation whereas NO-ASA suppressed enzymatic activity in a concentration-dependent manner with an approximate IC_{50} value of ~ 30 µM.

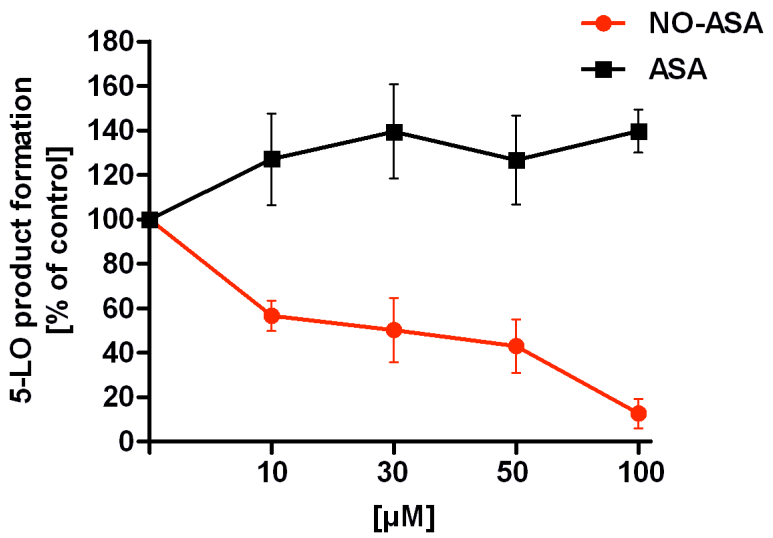

Fig. 51: *The influence of NO-ASA and ASA on 5-LO activity in intact PMNL in the absence of AA*

PMNL intact ($5x10^6$) were preincubated with ASA and NO-ASA for 15 min in the indicated concentrations. Cells were stimulated by the addition of 2.5 µM of A23187. Enzymatic activity was measured after stopping metabolite formation with cold methanol and extraction of the formed 5-LO metabolites followed by HPLC analysis. n=3. Values are expressed as mean + SEM.

In order to circumvent $cPLA_2$ signalling, AA was added as a natural substrate. Preincubation with drugs in indicated concentrations was performed for 15 min and cells were stimulated for upregulation of LT biosynthesis by the addition of A23187 (2.5 µM) and AA (20 µM) was added. After 10 min, catalytic activity was stopped by methanol and products were extracted and analysed by HPLC. In the presence of AA, nearly the same profile for both compounds evoked as in the absence of the natural substrate (Fig. 52). Again, ASA did not alter 5-LO activity. In contrast, NO-ASA showed concentration-dependent inhibition of 5-LO activity with an approximate IC_{50} value of ~ 30 µM.

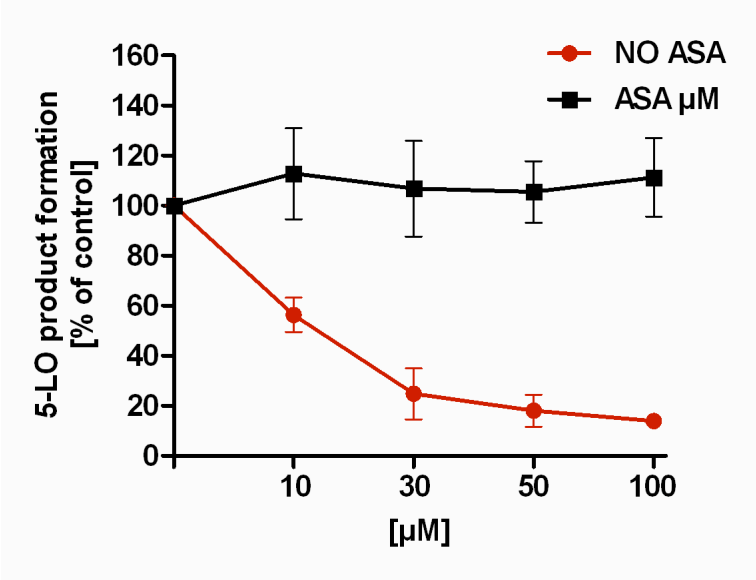

Fig. 52: *The influence of NO-ASA and ASA on 5-LO activity in intact PMNL in the presence of AA*

5x10^6 intact cells were pretreated with ASA and its NO-derivative for 15 min at 4°C. Stimulation was performed by 2.5 µM A23187 and 20 µM AA for 10 min at 37°C. 5-LO product formation was stopped yb the addition of methanol and 5-LO metabolites were analyzed by HPLC.n=4. Values are expressed as mean + SEM.

4.4.4 Inhibition of 5-LO product formation by NO-NSAIDs in cell free system

Considering the presented inhibition profiles of NO-sulindac and NO-ASA in the cell-based assay in the previous section, possible interactions of the other NO-NSAIDs were now assessed in a cell-free assay using purified human recombinant 5-LO. To identify probable interferences with the 5-LO enzyme and nitrosylated NSAIDs, drugs were incubated with the appropriate amount of protein leading to ~ 800 -1000 ng 5-LO products. Induction of 5-LO metabolites was elicited by the addition of Ca^{2+} (2 mM) and substrate was supplied exogenously (20 µM AA). The reaction was stopped after 10 min by the addition of methanol and analysis of 5-LO products was determined by HPLC. Interestingly, sulindac showed 5-LO enzyme inhibition of 50 %, but the nitrosylated derivative showed a more pronounced 5-LO suppression as only 20 % 5-LO actvitiy remained at 50 µM NO-sulindac. NO-derivatives of naproxen and ASA suppressed 5-LO product formation as well but less potent as compared to intact PMNL. Both compounds reduced 5-LO product formation levels only to ~ 40 % enzyme activity. Non-nitrosylated drugs lead to 5-LO activity between 60 % and 80 %. BWA4C was used as internal assay control (Fig. 53).

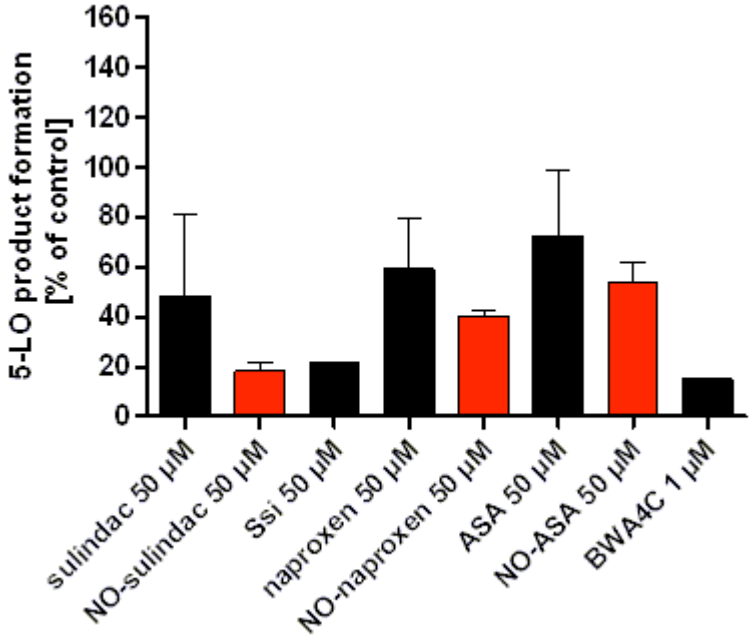

Fig. 53: *NO-NSAIDs and their influence on recombinant 5-LO activity*

Recombinant 5-LO protein was incubated with the indicated concentrations of sulindac, naproxen, ASA and their NO-derivatives for 15 min at 4C°. 5-LO activity was started by the addition of 2 mM Ca^{2+} and 20 µM AA. After product extraction, 5-LO metabolite levels were analyzed by HPLC.n=3. Values are expressed as mean + SEM.

4.4.5 5-lipoxygenase activity in human whole blood

Regarding suppressive capacities of 5-LO inhibitors, the question arises whether potential compounds with *in vitro* activity do show the ability to inhibit LT biosynthesis *in vivo*. Therefore, whole blood assays were performed. First experiments were carried out using the physiological stimulus LPS 10 µg/ml and fMLP 1 µM mimicking pathophysiological inflammatory conditions in the body. Freshly withdrawn venous blood was taken from healthy volunteers and studies were started immediately. After preincubation with substances for 30 min, samples were stimulated with the LPS/fMLP combination as described in the *material and methods* section. 15 min later, the reaction was stopped by placing samples on ice and plasma was obtained by centrifugation. Analysis by LC-MS-MS followed to quantify the formed LTs. All used drugs were applied at higher concentrations (100 µM) as tested before to exclude a possible lack of inhibition due to plasma protein binding or metabolism in the whole blood system. For determination of inhibtion pattern of the chosen compounds the 5-LO metabolites

LTB_4 and 5-HETE were detected and calculated. For all NO-derivatives almost the same suppressive efficacy occured as shown in Fig. 54. 5-LO product formation was inhibited by 50 %. Zileuton served as control inhibitor.

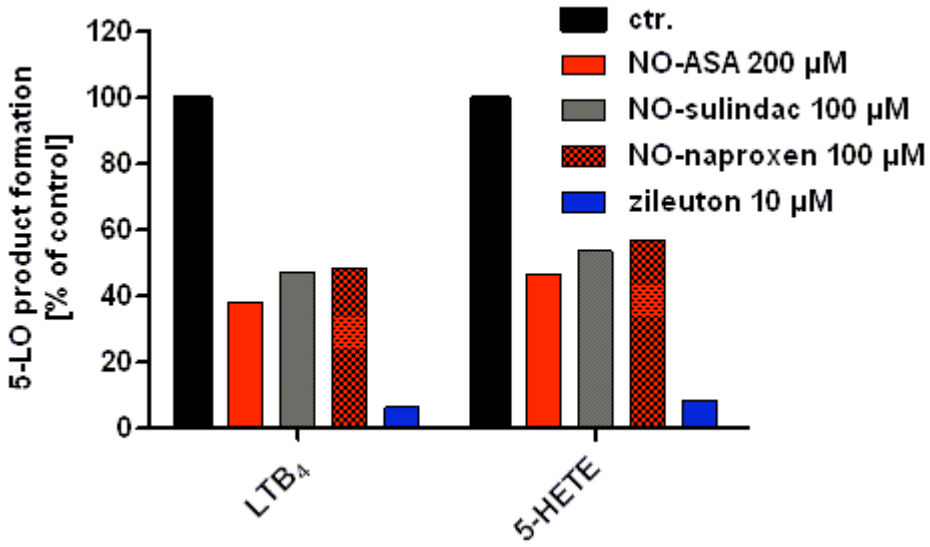

Fig. 54: *Effect of NO-NSAIDs on 5-HETE and LTB_4 formation in human whole blood*

Venous whole blood freshly gained from healthy volunteers was preincubated (30 min) with compounds at the concentrations indicated at 37°C. After the addition of the stimulus consisiting of LPS 10mg/ml and fMLP 1 µM reaction was performed for 1 h at 37°C and stopped by cooling the samples on ice. After obtaining plasma by centrifugation, LT levels were assessed by LC-MS-MS.Preliminary results, n=1.

4.4.6 Comments on future studies

Given the results from the previous section, it has clearly come out that NO-derivatives of certain NSAIDs could display a novel strategy for 5-LO modification and the released NO might directly interfere with the 5-LO enzyme. Further studies are needed to elucidate the molecular mechanisms involved in 5-LO suppression by NO-NSAIDs

5 DISCUSSION

The present results revelaed that Ssi is a novel 5-LO inhibitor with clinical relevance. 5-LO inhibition occured in stimulated leukocytes as well as with human recombinant purified 5-LO protein. Moreover, Ssi convinced in human whole blood assays via a potent suppression of 5-LO product formation. The molecular mechanism of the drug on 5-LO inhibition was investigated. The studies comprised elucidations of a possible influence of Ssi on 5-LO gene expression. Lastly, NO-releasing drugs were tested and evaluated on their 5-LO inhibitory potency. The following chapter therefore critically discusses the obtained data and places the findings into the so far published scientific background.

5.1 Suppression of 5-lipoxygenase product formation by Ssi

Sulindac is a well-studied NSAID and the general opinion is that sulindac's mode of action constitute an unselective COX-inhibitory activity, slightly preferential for COX-1. The drug also possesses analgesic, anti-pyretic and chemopreventive properties and is classified as a low-ulcerogenic NSAID that causes markedly less gastrointestinal toxicity as compared to related unselective COX inhibitors, such as diclofenac, indomethacin or naproxen [331]. Interestingly, sulindac exerts extraordinary properties among the class of NSAIDs concerning the overall severe side effect profile and leads therefore to less typical unwanted effects during continuous NSAID intake, such as dyspepsia, gastric ulcers or gastrointestinal bleedings. From the structural point of view the drug is ranked to the group of indene-acetates regarding its chemical profile und shows relations to the structure of indomethacin which is a strong inducer of gastrointestinal disorders.

Since the 1970s, more and more evidence evoked that the use of NSAIDs is linked to chemopreventive events and numerous experimental, epidemiologic and clinical studies suggest NSAIDs to play a promising role as anticancer drugs as they show induction of apoptosis and inhibition of angiogenesis, two strategies helping to suppress malignant tumor growth [332-335]. Several studies inidicated the chemopreventive use and their efficacy especially in colorectal cancer, but also in esophagus, stomach, breast, lung urinary bladder and ovary carcinomas where they may decrease the incidence of these types of cancer [333]. These findings have been

documented by four lines of evidence such as epidemiological studies, animal studies, *in vitro* experiments and clinical trials [336]. Liu *et al.* were among the first to show the involvement of COX-2 in tumorigenesis by observing an overexpression of COX-2 protein in colorectal and other tumors. It could be shown that the deletion of the COX-2 gene led to a decreased incidence of carcinomas in transgenic mice and so the rationale for the development of selective COX-inhibitors was born [337]. The link between COX-inhibition and chemoprevention was made as the unselective COX-inhibitor sulindac and the selective COX-2 inhibitor celecoxib confirmed their beneficial chemopreventive profile as they inhibited effectively the growth of adenomatous polyps and caused regression of existing polyps in randomized clinical trials [338-341]. However, there seemed to be COX-2-independent targets being responsible for the behaviour of these inhibitors, exemplified by celecoxib. Additional pharmacologic activities were employed and among various targets there seemed to be cellular components responsible for the drug's molecular mechanism [12]. As AA-produced eicosanoids have been implicated to a variety of diseases including different forms of cancers, the focus shifted then more from COX-derived PGs to LO-derived LTs and the hydroxyeicosatetraenoic acids (HETE's) as they exert also profound biological effects on the development of cancers and recent lines of evidence suggest that 5-LO products are especially involved in colon carcinogenesis [37, 38]. Two studies found that 5-LO inhibitors act chemopreventively in animal lung carcinogenesis when the FLAP inhibitor MK-886 and the 5-LO inhibitor A79175 were used in a mouse model [342, 343]. Anderson *et al*. showed reduced DNA synthesis and cell growth in human prostate cancer cells [344]. In this regard and having in mind the above mentioned, it was shown that increased LTC_4 production in gastrointestinal tissues was present and also a determinant for NSAID-induced ulcerogenicity and concomitant application of a 5-LO inhibitor abolished the indomethacin-induced gastrotoxicity in pigs [345]. As the molecular mode of action of chemopreventive-acting NSAIDs is only partly understood, it seemed most reasonable to elucidate further targets of the NSAID sulindac and molecular mechanism considerations evoked interference with the 5-LO pathway. The literature provides indirect evidence that 5-LO is inhibited by Ssi in humans. On the one hand, Ssi displays strong chemopreventive activities against colorectal cancer, which may be only partly attributed to inhibition of PG synthesis [346]. Moreover, 5-LO products participate in crucial tumor development events and pharmacological inhibition of 5-LO attenuates the growth of adeno-

matous colonic polyps in animals and potently induces antiproliferation and apoptosis of cultured tumor cells [347-349]. Thus, also the favourable gastrointestinal profile of sulindac may relate to its 5-LO inhibitory activity. In addition, the superior efficacy of Ssi against acute gout [350] may be associated with the drug's 5-LO suppressive activity since urate crystals stimulate the release of LTs from PMNL and may thereby contribute to the pathogenesis of the inflammatory reaction [351].

The first result section showed indeed potent inhibition of 5-LO product formation by sulindac's active metabolite, Ssi, in intact PMNL with calculated IC_{50} values of ~ 10 µM. In line with findings in literature, where Ssi displays the efficient compound, only Ssi inhibited 5-LO in PMNL, neither the prodrug sulindac nor the formed metabolite sulindac sulfone showed inhibitory potency on 5-LO. On cellular level natural substrate concentrations are important for enzyme performance as a competition may occur whilst inhibitors are tested. However, Ssi inhibited 5-LO product synthesis in PMNL equipotently regardless of whether AA was provided from endogenous sources via $cPLA_2$ or exogenously added to the reaction mixture. This leads to exclusion of an interference of Ssi with AA supply (refer to 4.1.1). 5-LO activation induced by intracellular Ca^{2+} influx was performed by challenging the leukocytes by A23187, one of the strongest chemical stimuli, capable of inducing 5-LO product biosynthesis circumventing receptor signalling [250, 352]. In terms of selectivity and regarding 12- and 15-LO it was found that Ssi only inhibits 5-LO very potently but did not affect 12- and 15-HETE production which is eligible as 15-HETE might interfere with the progression of malignancy and promotes the anticarcinogenic metabolism of AA [353, 354]. 12-LO products are connected to pro-carcinogenic events in the AA pathway and promote tumor progression and upregulation of synthesis in different types of adenocarcinomas are related to increased tumor growth [355-357]. Actually, inhibition of 12-LO products seemed preferable but as many different reports assign contrary properties to 12-HETE in the past years, 5-LO inhibition by Ssi is more favourable. In this regard, Ssi displays a selective inhibition profile on 5-LO.

5-LO is a tightly controlled enzyme with multiple regulatory mechanisms. Hence, in addition to direct inhibition of 5-LO, cellular components or mechanisms such as $cPLA_2$, FLAP, MAPKs, Ca^{2+} mobilization, interaction with coactosin-like protein and nuclear membrane association modulate 5-LO activity and may represent potential targets for Ssi [145]. Suppression of cellular product formation by an inhibitor could be caused by interactions at any of the multiple regulatory nodes on cellular 5-LO

activation and have to be considered and investigated when characterizing a novel inhibitor of 5-LO. Stress inducing stimuli like the pathophysiologically GPCR ligand fMLP in combination with bacterial LPS or SC and SA are mediating cellular LT activation by interference with MAPK pathway (activation of ERK 1/2 and p-38 MAP kinases) and are valuable conditions for a test system as the situation in the human body is closely mimicked. Ssi shows stimulus-independent efficacy to inhibit 5-LO in cells stimulated with fMLP 1 µM after priming with LPS 1 µg/ml and Ada 0.2 U/ml possibly excluding interference with the kinase signalling pathways. Different stress situations did not lead to siginificant differences in IC_{50} values of Ssi in cellular based assays, only a slight increase is visible while treated the cells with SA and SC compared to A23187. Seemingly, Ssi does not target any cellular regulating factors such as AA-providing $cPLA_2$ or MAPK pathways. As the next promising test system, assays with the purified 5-LO protein emerged reasonable. In line with previous results suggesting no interference of Ssi with any cellular components, the obtained results showed that purified recombinant human 5-LO is affected by Ssi as the efficient metabolite tested in cell-free assays showing ~ 80 % enzyme inhbition. Additionally tested sulindac and sulindac sulfone produced a maximal enzyme inhibition of ~ 20 %. This leads to the conclusion that only Ssi directly interferes with the 5-LO protein. As many factors influence cellular LT biosynthesis and 5-LO activity, further targets had to be adressed in a cellular context. In terms of the results in intact cells and cell-free assays, Ssi acted more potent in intact leukocytes with an IC_{50} of ~ 10 µM compared to purified 5-LO where 50 % inhibition was observed at ~ 20 µM Ssi. Therefore, as interference with different 5-LO activating pathways are unlikely for Ssi, inhibition of FLAP remains an alternative mechanism. Ssi exhibits structural similarity to the FLAP inhibitor MK-886 and Ssi suppressed the translocation of 5-LO in PMNL from the cytosol to the nuclear envelope which is an established property of FLAP inhibitors [322]. A similar inhibiton pattern can be as well recognized with the first introduced dual COX-FLAP inhibitor, licofelone (ML-3000), which prevented the nuclear redistribution of 5-LO in ionophore-activated PMNL as observed for other FLAP-inhibitors. The literature provides evidence that FLAP-inhibition was concluded after transfection experiments in HeLa cells were 5-LO inhibition was detectable only with co-transfected FLAP [323]. However, when FLAP was efficiently blocked by MK-886 in PMNL, Ssi still inhibited (FLAP-independently) 5-LO product formation in intact PMNL. Moreover, FLAP-dependent 5-LO inhibiton could be excluded since competi-

tion of Ssi with the FLAP-inhibitor MK-591 did not result in enhanced suppression of 5-LO enzyme activity. This leads to the conclusion that FLAP is not target of Ssi. Many inhibitors tested in isolated leukocytes with high efficacy *in vitro*, unfortunately failed to efficiently interact with LT biosynthesis *in vivo*. As the success and the beneficial profile of a newly discovered inhibitor for a potential application in humans depend on its efficacy and safety *in vivo*, studies imitating human body conditions are inevitable.Therefore, tests on Ssi's influence on LT production in human whole blood system were undertaken with freshly withdrawn blood from healthy volunteers and Ssi showed potent inhibition regardless which stimulus was used (LPS/fMLP or A23187). IC_{50} values of ~ 25 µM may appear high compared to inhibitors adressing their target in the nanomolar range. Thus, concentrations of Ssi achieved in humans with a bis daily intake of 400 mg Ssi peak at 36.8 µM and steady state concentrations as well range at ~ 25 - 30 µM. Results showed promising effects [358]. 5-LO inhbition of Ssi in human whole blood is almost comparable to COX-inhibition as the potency of Ssi to suppress COX-2 (IC_{50} ~ 4 µM) in whole blood was verified [359]. Namely, IC_{50} values differ from ~ 4 µM for COX isoformes, COX-1 and COX-2, and ~ 20 µM for 5-LO enzyme in human whole blood. Notably, inhibition of cellular 5-LO product synthesis occurred at lower Ssi concentrations with an IC_{50} ~ 8 -10 µM. This value is similar to IC_{50} values of Ssi for COX-2 (11 -14 µM) in transfected COS-1 cells [360]. Accordingly, 5-LO is a target of Ssi with almost equivalent susceptibility as COX-2 supported by the whole blood results, where Ssi suppressed the LPS-induced 5-LO product formation with an IC_{50} of 18.7 µM closely to the published IC_{50} value for COX-2 (10.43 µM) [359]. Despite these differences, almost full enzyme inhibition of both AA metabolizing enzymes COX and 5-LO is visible leading to a comparable efficacy at high Ssi concentrations (100 µM Ssi) in LPS-stimulated leukocytes. These findings of maximal suppression of 5-LO product synthesis by Ssi 100 µM display relevance for the previously hypothesized COX-independent mechanism of Ssi. Numerous COX-independent molecular mechanisms of Ssi have been observed *in vitro,* which may explain the drug's peculiar and beneficial pharmacological profile. Mechanisms like inhibition of the NF-κB activation by 200 µM Ssi, disruption of PPAR-δ signalling by 100 µM Ssi are described and the *in vivo* relevance of these mechanisms is debated because Ssi plasma concentrations >100 µM are not achieved in humans [361]. After ingestion of 400 mg (the recommended dose for treatment of arthritis), plasma drug concentrations reach a maximum of 38.6 µM, depending on the dosing

procedure [60]. The inhibition data in conjunction with the pharmacokinetic data suggest that standard dosage regimes of sulindac could reduce 5-LO product formation by more than 50 %. Importantly, pharmacokinetic studies demonstrated a prominent accumulation of Ssi in colonic epithelial cells raising the possibility of still even greater 5-LO suppression in these tissues [362].

Summary of 5-lipoxygenase inhibition by Ssi

To preliminary summarize Ssi's inhibitory mechanism on 5-LO, the presented results demonstrate that clinically relevant concentrations of Ssi suppress 5-LO product synthesis in different cellular assays including human isolated PMNL, triggered by either the physiological stimuli LPS/fMLP, but also by A23187 or cell stress, as well as in LPS/fMLP- and ionophore-stimulated human whole blood. Currently, according to the described data, sulindac represents the first functional COX/5-LO inhibitor on the market with 5-LO suppressive efficacy in whole blood at clinically relevant concentrations and a favourable tolerability in humans. In conclusion, it could be shown that the pharmacologically active metabolite of sulindac, Ssi, suppresses 5-LO product synthesis in leukocytes and in human whole blood at clinically relevant concentrations by direct interference with 5-LO. Suppression of 5-LO product formation may contribute to the pharmacodynamic profile of sulindac in animals and humans. Clinical studies that address the effects of sulindac on LT biosynthesis in patients at standard dosage regimes for prolonged periods would be a helpful expansion of the pharmacological actions of this drug. The obtained data suggest that Ssi suppresses cellular 5-LO product formation via direct inhibition of 5-LO, with the possible contribution of additional mechanisms, such as inhibition of 5-LO translocation.

5.2 Interference of Ssi with the regulatory domain of 5-lipoxygenase

Considerable efforts have been made in order to develop safe and efficient 5-LO inhibitors. However, many compounds failed under *in vivo* conditions either due to adverse effects (interference with other biological processes or production of reactive radicals) or a loss of efficacy due to increased oxidative state and/or phosphorylation of 5-LO in inflamed tissues [281, 288]. Elucidation of the binding mode of Ssi to 5-LO is of high interest and warrants investigations from which may emerge high affine,

selective and safe inhibitors of 5-LO with *in vivo* efficacy. In successive studies the mechanism on Ssi on 5-LO enzyme was investigated. Previous results made a direct 5-LO inhibition by Ssi most evident and it was obvious that Ssi's inhibitory potency varied dramatically in different test systems regarding intact leukocytes and their 100.000xg supernatants, homogenates and recombinant purified 5-LO protein [328]. Comparing the IC_{50} values of intact cells (~ 10 µM) and purified, recombinant 5-LO enzyme (~ 25 µM) to cell homogenates of PMNL (~ 100 µM) and S100 (~ 70 µM) it seems that these differences in inhibitory potency of Ssi on 5-LO are significant and are eventually due to a regulating factor or naturally occurring constituents in cell homogenates. It was speculated that in pure enzyme preparations Ssi is able to reach its target and does not have to compete with this factor. First, experiments were undertaken to gain detailed information about any other cellular targets which could potentially be affected by Ssi in a cell which is activated by an inflammatory stimulus. As described previously, the cellular redox tone is of great importance for the enzyme's catalytic acitivity and can be modified by certain specific inhibitors [145, 294]. Furthermore, the intervention with or generation of reactive oxygen species (ROS) may play an important role. Ssi is thought to sensitize cells to oxidizing agents and drugs that affect mitochondrial functions, resulting in the production of ROS and death by apoptosis [363]. However, compared to ZD-2138, a non-redox type inhibitor of 5-LO which requires reducing agents such as DTT in broken cell preparations or S100 of PMNL to perform inhibition, 5-LO inhibition by Ssi is Gpx-independent. Thereupon, other pathways leading downstream to the 5-LO signalling or procuring pre-cursors were elucidated. PLD is an enzyme responsible for processing phospholipids to PA and choline. Inhibition of PLD leads to diminished amounts of DAGs which usually, in an agonistic way, stimulate 5-LO [233, 364]. Therefore, PLD could represent a potential target of Ssi. However, PLD was not inhibited by Ssi. Another mechanism of 5-LO activation being possibly repressed by Ssi and resulting in diminished 5-LO product formation could be interference with intracellular Ca^{2+} levels. Intracellular Ca^{2+} increase is a very rapid process and signal transduction follows immediately. Upon stimulation with various stimuli like A23187 or fMLP, intracellular Ca^{2+} levels increase significantly in cells participating in the inflammation process. However, analysis of Ca^{2+} release in fMLP stimulated PMNL by a fluorescence-based assay showed no difference between Ssi-treated and untreated cells. Thus, interaction with Ca^{2+} mobilization and subsequently reduced 5-LO activity is not

likely. Another assumption may be an interaction with cellular 5-LO product synthesis by blocking the activation of MAPK, which phosphorylates and thereby stimulates 5-LO [145]. Interference with MAPK signalling by Ssi is unlikely since PMNL activation with A23187 widely circumvents receptor-coupled signalling and thus employs MAPK/phosphorylation-independent 5-LO activation [321]. On the other hand, Ssi was most potent against 5-LO when product synthesis was induced by receptor-coupled pathways (using fMLP and/or LPS as a stimulus) mediated by MAPK. Hence, under these conditions, an interference of the drug with the downstream receptor signaling pathways or the release of AA can not be excluded. In Western blot analysis, a possible Ssi-mediated ERK regulation was investigated from PMNL preparations. It was demonstrated that 5-LO activation by phosphorylation via MAPK is not affected by Ssi as ERK- and phosphorylated ERK protein patterns did not differ. Then other strategies were followed focusing on the purified protein and its structural features. As shown before, Ssi directly inhibits purified 5-LO enzyme and inhibition seems to be mediated by an interaction with the C2-like domain of the protein. This was first supported by the observation of Ssi's different potencies on 5-LO inhibition depending on the cellular environment where a loss of inhibitory potency occurred in reaction mixtures containing cellular membrane constituents (PMNL homogenates and S100). On the basis of these findings, an interference of 5-LO with the lipid binding site of 5-LO was suggested. The 5-LO enzyme, which translocates to the nuclear envelope upon cellular stimulation, forms LTs at the nuclear membrane where AA is provided by $cPLA_2$ and FLAP facilitates access to its natural substrate [148]. Membrane binding is mediated by trp residues within the C2-like domain of 5-LO and this lipid binding site, moreover responsible for the binding of PC liposomes, displays a possible target motif for Ssi. [211]. As inhibition of 5-LO by Ssi was supposed to occur via the mentioned trp amino acids, a competitive effect of the 5-LO inhibition and the addition of microsomal preparations consisting of membrane particles was explored. Microsomes, prepared of leukocytes and mimicking cellular membranes, were able to reverse Ssi induced 5-LO inhibition when added to crude 5-LO incubations. As there is evidence for hyperforin's binding mode via the lipid-binding sites, microsomes should occupy the predicted binding site and in consequence inhibition of 5-LO should fail. Presented results could support this hypothesis. It has been shown in the published literature that phospholipids like PC can stimulate 5-LO by interfering with the regulatory C2-like-domain of the enzyme [210]. These

interactions are mediated by an interference with the PC selective, membrane association modulating, three amino acid residues located on position 13, 75 and 102 of the regulatory domain of 5-LO [205, 213]. These binding properties are distinguished by the activation factor Ca^{2+} thus stimulation of the enzyme can occur [205]. PE and PS function as constituents of natural biomembranes but lack the ability of binding to conserved regions of C2 domains or C2-like domains [211]. In present investigations, uniquely significant interactions with PC were detectable whereas other lipids (PE or PS) did not show considerable impact on 5-LO inhibition by Ssi. These findings could not be observed with the control inhibitor BWA4C. Hyperforin displayed the same profile as found for Ssi so far. It seemed reasonable, that a 5-LO protein mutant lacking three trp residues which are essential for intact functionality and target the 5-LO enzyme to nuclear membranes, should not be affected by Ssi. Due to the mutation within the regulatory domain of the 5-LO enzyme, the binding properties of 5-LO-3W should account for reduced lipid binding. Subsequently, diminished stimulation of enzyme activity should be observable [216]. The mutated protein (5-LO-3W) where trp residues are replaced to ala is known to show lower response to PC liposomes induced activity enhancement [210]. Given that binding of Ssi is mediated via the three corresponding amino acids, it was expected that Ssi would not induce decreased 5-LO product formation whilst added to the 5-LO-3W mutant. Indeed, in the present work almost no influence of Ssi on this 5-LO-3W mutant was observed while the 5-LO-wt was inhibited in a concentration-dependent manner. This leads to the conclusion that these amino acids are required for accessibility and inhibition of 5-LO by Ssi. BWA4C potently suppressed the 3W mutant 5-LO protein supporting the hypothesis identifying Ssi as a novel approved drug inhibiting 5-LO by interference with the lipid binding site within the regulatory C2 like domain. For 5-LO inhibitor categorization (refer to section 1.2.9 for details) three groups can be built up: redox-active compounds interfering with the non-heme iron of 5-LO in the catalytic domain, iron-chelating substances and fatty acid mimetics, the so called non-redox type inhibitors, which act in a competitive manner with the natural substrate of 5-LO [146]. However, Ssi did not show iron-chelating properties and a direct interaction for example reducing the iron atom or radical scavenging could not be demonstrated (data not shown). A competitive mode of action could be excluded as previous studies indicated an uncompetitive mode of inhibition of 5-LO [328]. Results obtained with purified, isolated 5-LO seemed to confirm a direct interaction with the 5-LO protein. Efforts to deter-

mine a selective binding via a linking with the putative binding site holding the three essential trp residues located in the C2-like domain were carried out using the saturation-transfer-difference NMR approach. The studies showed clearly binding of Ssi to the 5-LO-wt but unfortunately affected the 5-LO-3W as well (data not shown), probably due the high Ssi concentrations (160 - 200 µM) needed for NMR analysis and therefore resulting in unspecific binding. However, a second possible binding site on 5-LO possibly occupied at higher concentrations of Ssi will lack physiological relevance for the drugs application as only 38.6 µM plasma drug concentrations are achieved after standard uptake and concentrations used lastly for NMR studies ranged from 100 – 200 µM [60].

Concluding remarks on Ssi's molecular mechanism on 5-lipoxygenase

Thus, Ssi potently inhibited purified 5-LO enzyme but lost efficacy when assays are performed in different cellular systems, especially differences in inhibitory potencies in intact cells and isolated 5-LO compared to cell homogenates and S100 preparations are evident. Microsomes, added to isolated 5-LO protein diminished the efficacy of Ssi presuming a competitive action of the inhibitor with lipid particles. PC liposomes were capable of counteracting of Ssi's 5-LO inhibitory effect. All these facts led to the presumption of an interference of Ssi with the membrane targeting site located in the C2-like domain of the 5-LO enzyme. This could be supported by inhibition patterns of Ssi on a 5-LO protein mutant lacking the responsible trp residues. As increasing evidence suggests a role for 5-LO products found in a variety of cancer tissues, inhibition of 5-LO enzyme activity presents a strategy for chemopreventive intervention [4, 38]. Ssi possesses certain chemopreventive properties and by unknown mechanisms inhibits tumor cell growth and reduces polyp development in colorectal cancer which maybe attributed to reduced LT biosynthesis in these tissues [347, 349]. Other COX-2 selective inhibitors where COX-independent effects have been attributed did only show modest reduction regarding polyp growth in FAP patients in contrast to Ssi [365]. As Ssi lacks severe side effects manifesting in lower allergenic toxicity and beneficial cardiovascular profiles, the mechanistic analysis of the inhibition mode of Ssi in 5-LO product formation is of great importance and may provide answers explaining the peculiar and beneficial profile of the drug [331]. Collectively, the present work displays a novel strategy inhibiting LT formation by block-

ing the early step in LT biosynthesis by Ssi targeting the C2ld of 5-LO. The identification of Ssi's target structure on 5-LO offers the possibility of designing potent compounds showing even more promising chemopreventive efficacy and favorable profiles in the treatment of cancer. Taken together, Ssi is the first approved drug on the market exhibiting 5-LO inhibition by blocking the regulatory domain of the enzyme by a direct interaction. Therefore Ssi could represent a novel lead structure for 5-LO inhibitors.

5.3 Ssi's influence on 5-lipoxygenase expression

In the early 1990ies, the role of COX-2 expression was evaluated and correlated to enhanced tumor growth using a transgenic mice model. Furthermore, it was concluded that overexpression of COX-2 alone was sufficient to induce tumorigenic transformation in a tissue-specific manner [337]. It was possible to establish a strong correlation between the use of NSAIDs and the decreased incidence of colorectal, breast and lung cancers [334, 366, 367]. Carcinomas of different origins have been investigated and a COX-2 upregulation was found to occur in 85 % of all human colon carcinomas and 50 % in colon adenomas, supported by gene deletion studies [61, 368-370]. However, there is a considerable body of data supporting the hypothesis of COX-independent targets of NSAIDs being responsible for chemopreventive and antitumorigenic actions [61]. The literature provides evidence that molecular mechanisms of several NSAID-mediated anti-cancer effects can be attributed to interference with inhibition of COX-2 as target gene [371, 372]. It is well established that other genes are described to be altered by drugs and chemicals by increased or decreased gene expression and are as well involved in mechanisms of cancer prevention independent from PG formation. One example is the NSAID-activated-gene-1 (NAG-1) which belongs to the TGF-ß superfamily of growth factors and is regulated by several NSAIDs [373]. Both, COX-selective and COX-unselective inhbitors increase NAG-1 expression. Structural characteristics for COX inhibition seemed to be different from the regulation of NAG-1 and prevention of cancer activity. In former publications it was reported that Ssi possessed the most prominent properties to induce NAG-1 gene expression as ample inductions were observed at concentrations at 1 - 50 µM, a physiological concentration achieved *in vivo* [373]. It has not been published yet that 5-LO expression is downregulated by chemopreventive-acting

NSAIDs. As a working hypothesis it was speculated whether 5-LO displays such a target gene for NSAIDs and if Ssi contributes to the regulation of 5-LO on transcriptional level. Therefore, the studies comprised first analysis of a primary effect of Ssi on 5-LO gene regulation in the monocytic cell line MM6 after induction of cell differentiation and concomitantly cellular 5-LO synthesis. Cells were treated with Ssi and the obtained qPCR data showed a concentration-dependent decrease of 5-LO mRNA in differentiated MM6 cells after 72 hrs of incubation with Ssi. This decrease did not occur, as expected in control cells (undifferentiated). ß-actin was used as housekeeping gene control. Low concentrations (0.3, 1, 2 µM) of Ssi were able to suppress 5-LO mRNA formation to amounts half of the control, but higher concentrations led to only 20 % of initial copy numbers. These findings argue for an influence of 5-LO m-RNA formation which could be mediated by a regulating element such as a transcriptional factor. As refered to in previous sections (1.2.6), the 5-LO promoter region consist of five repeated GC-rich sequences, discovered as transcription factor binding region, where Sp1, a common transcriptional regulatory factor, can bind [173]. Sp1 regulates basal transcription of NAG-1 gene through certain GC-boxes [374]. As Sp1 is known to show cabability to bind on these promoter fragments residing in the 5-LO promotor and is also required for 5-LO expression in monocytes, a possible interplay of Sp1, 5-LO and Ssi could be conceivable. Realtime PCR results from the same experimental approach as aforementioned showing a reduced mRNA level of Sp1 while treated with Ssi underlined the possible involvement of Sp1 in Ssi-induced mRNA regulation of 5-LO [175, 179]. More information about Sp1's involvement in the downregulation of 5-LO mRNA by Ssi was gained in reporter gene assay where Sp1 was cotransfected and its promoter activating property was at least partly counteracted by increasing concentrations of Ssi. A ChIP assay could underline Sp1 as a possible binding partner involved in the Ssi-mediated downregulation of 5-LO at a concentration of 30 µM Ssi which is physiologically relevant. Recently, p53, a tumor suppressor gene, responsible for the induction of NAG-1 which is linked to cell death, could be another probable player in the outlined scenario, but this remains to be elucidated [375].

Concluding remarks

A shift in AA metabolism by the use of COX-inhibitors may be a mechanism warranting further consideration for contribution to the prevention of cancer. The reported findings support the conclusion that the chemopreventive activity of unselective COX-inhibitors such as sulindac and its active metabolite Ssi, is mediated by alterated gene expression. For example, COX-2 and NAG-1 gene are well documented genes involved in cancer preventive activities of COX - inhibitors. The description of the 5-LO gene as a novel target being downregulated by Ssi may, with the view to 5-LO and its target functions in general concerning several types of cancer, demonstrate a novel strategy for screening potential anticancer drug candidates. Ssi may stand for a novel structure of 5-LO inhibiting compounds additionally showing gene regulatory functions and therefore possesses highly beneficial profiles for the use in humans.

5.4 Modulation of 5-lipoxygenase enzyme activity by NO-NSAIDs

Chemoprevention is a wide spread field and great efforts have been made to gain more understanding of the behind lying molecular mechanisms. New drug development strategies have been made to produce safer and more efficient drugs, ideally combining two modes of action or operating simultaneously at two targets. Even though NSAIDs are promising cancer chemopreventive agents and long-term use of aspirin and other NSAIDs have been shown to reduce the risk of cancer of the colon and as well as of other cancers of the breast, prostate, lung and skin, disadvantages like gastrotoxicity still persist. Despite the exploration and knowledge provided by continous research and substantial insights into the mechanism by which NSAIDs modulate these events, questions still need to be answered with regard to safety, efficacy, optimal treatment regimen and mechanism of action [376]. In search of all these matters, discovering NO as a molecule with gastroprotective properties and the ability to mimick the effects of PGs, this emerges the rationale for the development of NO-releasing NSAIDs. Synergistic effects were suspected from the COX-inhibition on the one hand and the gastroprotective effect in terms of ulcerogenicity on the other hand. It seemed of major relevance to investigate and to compare possible inhibition patterns of compounds and their NO-releasing partners on 5-LO activity and therefore maybe to identifiy the molecular mechanism of these drugs. Several NO-NSAIDs were currently under investigation such as NO-naproxen, the first agent of this novel

class of drugs named COX-inhibiting nitric oxide donators (CINOD) [75]. NO-asprin and NO-sulindac can also be classified into this group of drugs [66]. In search of new concepts of 5-LO inhibiton, a third working hypothesis was therefore based on the previous results, where Ssi as a conventional NSAID showed the ability to inhibit LT biosynthesis by direct interference with the 5-LO enzyme. It was assumed that thereby NO-NSAIDs justify their chemopreventive activity in certain tumors. As shown in previous sections, sulindac, a prodrug, did interfere with 5-LO activitiy neither in cellular systems nor in cell free assays. In the case of NO-sulindac, it was suspected that an appropriate inhibition of 5-LO enzyme activity can only take place if NO, released from the nitrosylated sulindac, interferes in any mode of action with the protein structure. Examinations with NO-sulindac and NO-naproxen in cellular systems such as PMNL evoked inhibition of 5-LO only by the NO-derivative, but not by the non-nitrosylated parent NSAID. A dose-response curve of NO-sulindac exerts the most potent inhibitory profile (IC_{50} ~ 2 µM) which could be confirmed in studies on the recombinant purfied enzyme where NO-sulindac acts even more potent than Ssi. The obtained results led to the conclusion that NO may be a key factor involved in LT biosynthesis suppression. As NO-NSAIDs were developed, a straightforward mechanism was outlined which involved the release of cytoprotective NO being responsible for the decreased mucosal damage in the stomach usually caused by the regularly intake of NSAIDs from NO-NSAIDs [63]. This concept is widely discussed in the literature and NO is appreciated to play a double role being able to protect, but also to promote cancer [377]. The question raised whether it is NO that brings the cytoprotective and/or anticancer effect. It was thought that NO is being released in the upper gastrointestinal tract to elicit its protective properties [378, 379]. The strongest evidence against this hypothesis is found in the fact that NO-aspririn, the most prominent NO-NSAID, passes the stomach without breakdown as the drug was detected in the duodenum after intake [72, 73]. Indeed, a possible explanation could be that NO reaches the mucosa via the circulation site as it is speculated that NO is cleaved by breakdown of the drug when it reached the circulating blood stream and additionally elevated NO levels have been reported after NO-NSAID administration using NO-flurbiprofen in a rat modell [72, 380]. The obtained inhibition pattern of NO-ASA beside the use of ASA as control, which is known to show no interaction with the 5-LO pathway, however evoked almost the same picture. Only the NO-derivative was active on 5-LO enzyme. Suppression by NO-ASA was rather potent and substrate-

independent with IC_{50} values of ~ 30 µM in the cellular assay provoking a non-competitive mode of action. A slight increase in the IC_{50} values (~ 50 µM) was found in cell-free assays presuming additional cellular components supporting 5-LO inhibition by NO-ASA. In literature, mechanistical studies are mostly found about NO-ASA proposing effects on MAPK pathway, inhibition of iNOS and inhibition of NF-kB activation [81-83]. To complete the preliminary data, whole blood studies showed suppression of LT biosynthesis products LTB_4 and 5-HETE as well but rather low suppressive potential was observed as all compounds used (NO-sulindac, NO-naproxen, NO-ASA) only inhibited 5-LO enzyme activity up to 40 - 50 % residual enzyme activity. Extensively higher concentrations were needed to gain this inhibition pattern compared to cellular-based and cell-free assays. As an explanantion could serve either an insufficient NO-release into the blood stream or that the amount of NO is not sufficient to achieve 5-LO inhibition. Probably, these findings are due to the fact that released NO is promptly bound by plasma proteins in the circulation blood stream. However, a direct intereference with one of the regulating parts of the 5-LO protein seems likely for the NO-derivatives NO-ASA, NO-sulindac and NO-naproxen as all drugs together show the ability to suppress the purified recombinant 5-LO enzyme. As mentioned in the future comments of the result section 4.3, a broad spectra of experiments has to be carried out to clarifiy the detailed mechanism underlying the interaction of NO-NSAIDs and 5-LO.

Concluding remarks

The last section should verify, whether a link between the chemopreventive efficacy and beneficial gastrointestinal profiles of NO-NSAIDs and 5-LO as a molecular target can be built up. NO-NSAIDs such as NO-sulindac, NO-aspirin or NO-naproxen were able to inhibit 5-LO enzyme activity, whereas NO-sulindac showed the most potent inhibition pattern regardless whether tests were performed in cellular assays or cell-free assays. Only in human whole blood systems all compounds showed almost equipotent inhibitory potency on 5-LO. It is still a matter of debate whether the NO molecule is required for the chemopreventive, antitumorigenic and gastroprotective properties which is ascribed to NO-NSAIDS. The present results link NO-NSAIDs to the 5-LO pathway and could provide evidence for 5-LO as a novel target of NO-NSAIDs.

6 SUMMARY

During the last years, chemopreventive activity of NSAIDs against a great variety of tumors was highly investigated. COX-2 seemingly plays a major part in tumorigensis and tumor development, underlined by several studies in animals and humans. At first, NSAIDs were thought to accomplish chemoprevention by inhibition of COX-2 as their so far known mode of action comprises unselective inhbition of COX-enzymes. However, further studies revealed COX-independent mechanisms. Sulindac is known as a well established drug used to treat inflammation and pain exerting the most prominent chemopreventive action, mainly in colorectal cancer or FAP and can be classified into the group of NSAIDs inhibting both COX-isoformes. As interference with the AA metabolism is evident, it was speculated whether Ssi has targets other than COX-enzymes providing evidence and explanation of its beneficial side effect profile and its ability to reduce tumor growth. 5-LO is another master enzyme in the AA cascade which produces inflammatory lipid mediators (LTs) upon stimulation in inflamed tissues. The present work should answer the question if Ssi targets the 5-LO pathway and should examine the molecular mechanisms behind Ssi-mediated 5-LO inhibiton. As COX-2 is upregulated during carcinogenesis and is inhibited by Ssi, further investigations should show regulatory effects of Ssi on 5-LO gene expression in MM6-cells and whether Sp1 as a common transcriptional factor is involved in such a regulation. As the use of NO-NSAIDs seem to be a promising strategy concerning their chemopreventive and gastroprotective effects compared to the parent NSAIDs, a possible interaction with the 5-LO pathway as a second, potent target should additionally be elucidated. In the first section it was demonstrated that the pharmacologically active metabolite of sulindac, Ssi, targets 5-LO. Ssi inhibited 5-LO in ionophore A23187- and LPS/fMLP-stimulated human PMNL ($IC_{50} \approx 8$ -10 µM). Importantly, Ssi efficiently suppressed 5-LO in human whole blood at clinically relevant plasma levels (IC_{50} = 18.7 µM). Ssi was 5-LO-selective as no inhibition of related lipoxygenases (12-LO, 15-LO) was observed. The sulindac prodrug and the other metabolite, sulindac sulfone, failed to inhibit 5-LO. Mechanistic analysis demonstrated that Ssi directly suppresses 5-LO with an IC_{50} of 20 µM. Together, these findings may provide a novel molecular basis to explain the COX-independent pharmacological effects of sulindac under therapy. In the second part of the work dealing with the analysis of Ssi's inhibitory mechanism on 5-LO it was presented that Ssi shows a lack of potency in cellular systems where membrane constituents are existent. The addition of microsomal frac-

tions of PMNLto crude 5-LO enzyme were able to recover enzyme activity to ~ 100 %. Selectively 5-LO activity stimulating lipids like PC, participating in 5-LO membrane interactions within the regulatory C2-like domain of 5-LO, counteracted the Ssi-mediated inhibition on 5-LO-wt in a concentration-dependent manner. Lastly, a protein mutant lacking three trp resudies essential for linking the enzyme to nuclear membranes and deploying catalytic activity was not influenced by Ssi and shows enzyme activity in a cell-free assay. Ssi displays the first 5-LO inhibitor on the market interacting with the C2-like domain of the enzyme and therfore can stand for a novel lead structure of 5-LO inhibitors. An influence on 5-LO gene expression by Ssi could be detected in differentiated MM6-cells, described in the results chapter 3 (4.3). Ssi downregulated the 5-LO mRNA level after 72 hrs of incubation in differentiated MM6-cells to ~ 20 % of output control at concentrations of 10 µM. Concomitantly, mRNA levels of Sp1 were suppressed. Reporter gene studies revealed Sp1 most probably as a regulating agent involved in the Ssi-mediated 5-LO mRNA downregulation as co-transfection of increasing amounts of Sp1 could abrogate the effect. A ChIP assay could identify Sp1 as a critical transcriptional factor as Sp1 binding to the 5-LO promoter decreased in presence of Ssi. Lastly, three NO-NSADIs (NO-sulindac, NO-naproxen, NO-aspirin) were tested for the ability of 5-LO product inhibition. In intact PMNL, all compounds showed effective inhibition of 5-LO activity and NO-sulindac was most potent with an IC_{50} value of ~ 3 µM. NO-ASA inhibited 5-LO with IC_{50} values of ~ 30 µM and showed a non-competitive mode of action in cell-based assays. On human recombinant 5-LO all compounds again showed inhibitory potency whereas NO-sulindac again suppressed LT biosynthesis with an IC_{50} vaue comparable to intact cellular systems. Unfortunately, all inhibitors showed a loss of potency when tested for inhibition of 5-LO product synthesis in human whole blood as higher concentrations up to 100 µM were needed to reach at least 55 % enzyme inhibition. However, this strategy of 5-LO inhibition seems promising and needs further experimental approaches to gain more insight into the mechanism of 5-LO inhibition by NO-NSAIDs.

7 ZUSAMMENFASSUNG

Die chemopräventiven Wirkungen von nichtsteroidalen Antirhheumatika (NSAR) erfuhren in den letzten Jahren reges Interesse und waren Gegenstand einer Vielzahl von Studien. In diversen Untersuchungen in Tiermodellen, aber auch in Humanstudien konnte gezeigt werden, dass das Enzym Cyclooxigenase-2 (COX-2) eine wesentliche Rolle bei der Tumorentstehung sowie in der Tumorentwicklung spielte. Durch Umsetzen des natürlichen Substrates Arachidonsäure (AA) entstehen im sogenannten Eicosanoid-Stoffwechsel die Prostaglandine (PG). In Tumorgeweben, vor allem bei kolorektalen Karzinomerkrankungen, sind eine Überexpression der COX-Enzyme sowie dementsprechend eine erhöhte PG-Synthese zu beobachten. Die durch enzymatische Aktivität der COX-2 synthetisierten PG stellen Trigger der tumorassoziierten Angiogenese dar, wirken modulatorisch auf das Immunsystem und regulieren Zellmigration und Zellinvasion. Die COX-2 stellte somit ein vielversprechendes Targetmolekül für die Krebsforschung dar. Da NSAR als primär bekannten Wirkungsmechanismus über eine unselektive Hemmung beider Isoformen der COX-Enzyme wirken, erklärte man sich auch die chemopräventiven Wirkungen dieser Substanzen über die Inhibition der COX-2-Aktivität. Jedoch lieferten viele Untersuchungen auch Hinweise auf COX-unabhängige Mechanismen. Sulindac, einzuordnen in die Gruppe der NSAR, stellt einen schon lange am Mark befindlichen und untersuchten Wirkstoff dar, der zur Behandlung von Schmerzen und entzündlichen Erkrankungen eingesetzt wird. Der bisher bekannte Wirkmechanismus des aktiven Metaboliten von Sulindac, Sulindac-Sulfid (Ssi), ist die unselektive COX-Hemmung. Unter den NSAR stellt Sulindac den potentesten Vertreter der chemopräventiven Wirkstoffe dar und wird hier vor allem zur Behandlung von familiärer adenomatöser Polyposis (FAP) und bei kolorektalen Karzinomerkrankungen eingesetzt. Da NSAR respektive Sulindac über die COX-Hemmung in den AA-Stoffwechsel eingreifen, war eine naheliegende Vermutung, dass Ssi auch andere Enzyme in diesem Stoffwechselweg beeinflusst. Dies könnte das nebenwirkungsarme Profil bezüglich gastrointestinaler Schädigungen sowie auch die chemopräventiven Wirkungen erklären. Die 5-Lipoxygenase (5-LO) stellt ein weiteres Hauptenzym im AA-Metabolismus dar und synthetisiert proinflammatorische Lipidmediatoren, die sogenannten Leukotriene (LT) nach erfolgter Stimulation der Zelle bei Entzündung. Die vorliegende Arbeite sollte nun untersuchen, ob Ssi mit dem 5-LO-Signalweg interagiert und möglicherweise einen Einfluss auf die LT-Biosynthese hat. Des Weiteren sollten mögliche zugrunde-

liegende molekulare Mechanismen aufgeklärt werden. Da die COX-2 in Tumorgeweben hochreguliert ist und durch NSAR eine Regulation der Expression erzielt werden kann, sollte anschliessend auch ein etwaiger Einfluss von Ssi auf die Expression des 5-LO Enzyms untersucht werden. Es stelle sich die Frage, ob Sp1, ein bekannter Transkriptionsfaktor, bei einer etwaigen Regulation im zellulären System in Mono-Mac-6 (MM6) Zellen beteiligt sein könnte. Eine neue, vielversprechende Strategie im Hinblick auf gastroprotektive als auch chemopräventive Eigenschaften stellt die Entwicklung der sogenannten Nitro-NSAR dar (NO-NSAR), welche im Vergleich zu den herkömmlichen NSAR einen NO-freisetzende Substituenten und die Struktur eines NSAR kombinieren, verbunden durch ein Spacermolekül. Da aufgrund der PG-Hemmung deren zytoprotektive Eigenschaften wegfallen, war es naheliegend, die vorteilhaften PG-Wirkungen durch im Gastrointestinaltrakt lokal freigesetztes NO zu imitieren. NO-NSAR zeigten im präklinischen Modell der Zellkultur und auch im Tiermodell chemopräventive Eigenschaften bei verschiedenen Krebsarten. Vorliegend sollte hier evaluiert werden, ob verschiedene NO-NSAR die 5-LO als Zielstruktur adressieren.

Im ersten Teil der Arbeit konnte gezeigt werden, dass das NSAR Ssi als ein potenter Hemmstoff der 5-LO agiert. Es stellte sich heraus, dass nur Ssi als der pharmakologisch wirksame Metabolit von Sulindac einen Einfluss auf die LT-Biosynthese aufwies, nicht jedoch das Prodrug Sulindac und der Metabolit Sulindac-Sulfon. Weitere getestete Verbindungen, die der Klasse der NSAR zuzuordnen sind (Diclofenac und Indomethacin) hatten keine inhibitorische Wirkung auf die Aktivität der 5-LO. Die Hemmwirkung von Ssi auf die 5-LO wurde werder durch die Abwesenheit noch durch steigende Mengen an natürlichem Substrat des Enzyms, der AA, in Konzentrationen von 2µM und 20µM verringert. Des Weiteren zeigte sich in der Produktanalyse frt 15-Lipoxygenase (15-LO), die auch in Granulozyten vorkommt, eine Selektivität von Ssi für 5-LO. Die in Plättchen vorkommende 12-Lipoxygenase (12-LO) wurde in ihrer Aktivität eher durch eine Behandlung der Zellen mit Ssi stimuliert, während Ssi keinerlei Einfluss auf die Produktbildung der 15-Lipoxygenase (15-LO) aus Granulozyten hatte. Ssi hemmte die 5-LO in sowohl mit Calcium-Ionophor (A23187) als auch mit LPS/fMLP (physiologischer Stimulus) stimulierten humanen Leukozyten (IC_{50} 8-10µM). Weitere Stimuli der 5-LO-Produktbildung, die zum einen osmotischen Stress der Leukozyten hervorrufen (Natriumchlorid) und zum anderen chemischen Stress der Zellen verursachen (Natriumarsenit) führten nicht zu signifikanten Unterschieden

der Inhibitorpotenz von Ssi. Auf Proteinebene konnten mechanistische Studien eine potente Hemmung der 5-LO-Aktivität nach Inkubation mit Ssi und anschliessender Stimulation des humanen, rekombinanten 5-LO-Proteins nachweisen (IC_{50} = 20µM). Hier zeigte sich wieder die bereits im zellulären System beschrieben Wirkung des aktiven Metaboliten Ssi an 5-LO, Sulindac und Sulindac-Sulfon und auch Indomethacin führten nicht zu einer Inhibition der 5-LO-Produktbildung. In Versuchen, die die subzelluläre Lokalisation des 5-LO-Proteins untersuchen sollten, stellt sich heraus, dass Ssi eine Hemmung der Translokation des 5-LO-Proteins zur Kernmembran vermittelt. Die Schlussfolgerung, dass dies in einer Hemmung des 5-Lipoxygenase-aktivierenden-Proteins (FLAP) durch Ssi begründet liegt, konnte jedoch in einem Kompetitionsassay mit einem bekannten FLAP-Inhibitor (MK-886) nicht bestätigt werden. Weiterhin supprimierte Ssi wirksam die 5-LO-Produktbildung im humanen Vollblutversuch in klinisch relevanten Konzentrationen (IC_{50} = 18.7µM) unabhängig vom verwendeten Stimulus (A23187 oder LPS/fMLP). Auch in diesem Testsystem erwies sich Ssi als wirksame 5-LO-inhibitorische Substanz während Sulindac und Sulindac sulfone keine suppressive Wirkung auf die 5-LO-Aktivität zeigten.

Im zweiten Teil der vorliegenden Arbeit, der sich mit der mechanistischen Aufklärung der Hemmung von Ssi an 5-LO befasste, wurde ein Verlust der Inhibitorpotenz in Abhängigkeit von der zellulären Umgebung festgestellt. In intakten Zellen und am Reinenzym erreichte Ssi IC_{50}-Werte von 15-25µM während in Zellhomogenaten und dem 100.000xg Überstand (S100) IC_{50}-Werte von 30-100µM vorlagen. Waren Membranbestandteile im Reaktionsansatz vorhanden, so wie etwa in Zellhomogenaten, verlor Ssi deutlich an inhibitorischer Potenz. Die Vermutung, dass die zelluläre Zusammensetzung einen Einfluss auf den Redoxzustand der 5-LO und somit auch für eine Hemmung verantwortlich ist, konnte nicht bestätigt werden. Zunächst wurden zelluläre Signalwege untersucht, auf die der Arzneistoff Ssi Einfluss haben könnte. Daher wurde ein potentieller Einfluss von Ssi auf die Phospholipase D (PLD) untersucht. Es zeigte sich jedoch keine durch Ssi verursachte Enzymhemmung. Da Calcium ein starker Stimulus für eine Aktivierung des 5-LO-Enzyms darstellt, wurde als nächstes die Freisetzung von Calcium in Leukozyten evaluiert, jedoch zeigte sich hier auch keine Veränderung durch Ssi im Vergleich zur unbehandelten Kontrolle. Im zellulären Kontext wurde eine Phosphorylierung und nachgeschaltet eine Aktivierung der 5-LO durch die extracellular-regulated-kinase (ERK 1/2), eine Kinase aus der mitogen-activated-protein-kinase (MAPK) Familie, beschrieben. Eine Hemmung von

ERK 1/2 durch Ssi wurde im Western Blot-Experiment jedoch nicht beobachtet. Daher fokusierten sich die folgenden Versuche auf Interaktionen von Ssi mit dem aufgereinigtem 5-LO-Enzym. Die Zugabe von aus PMNL gewonnen mikrosomalen Fraktionen, die membranäre Bestandteile in der Zelle immitieren sollten, zu 5-LO-Reinenzym in Gegenwart von Ssi konnte die Enzymaktiviät nahezu wieder auf 100% herstellen. Phospholipide, die ebenfalls Bestandteile von Membranen darstellen, vermitteln die für die 5-LO-Aktivität essentielle Membranbindung durch Interaktion mit der regulatorischen C2-ähnlichen Domäne des Enzyms. Insbesondere Phosphatidylcholin (PC) ist in der Lage, die 5-LO-Aktivität selektiv zu stimulieren und konnte die Ssi-vermittelte 5-LO-Hemmung konzentrationsabhängig aufheben. Andere Phospholipide wie Phosphatidylethanolamin (PE) und Phosphatidylserin (PS) zeigten weder einen stimulatorischen Einfluss auf die rekombinante 5-LO noch ließ sich die durch Ssi hervorgerufene Enzymhemmung aufheben. Um die Interaktion von Ssi mit der C2-ähnlichen Domäne der 5-LO zu verifizieren, wurde eine 5-LO-Proteinmutante generiert, bei der drei Tryptophanreste, die essentiell für die Membranbindung der 5-LO sind, gegen Alanin ausgetauscht wurden. Das mutierte Protein wurde von Ssi nicht beeinflusst und zeigte unverändert katalytische Aktivität. Dies spricht für eine Interaktion von Ssi mit den drei die Membranbindung vermittelnden Tryptophanresten. Ssi ist der erste 5-LO-Inhibitor auf dem Markt, der an der C2-ähnlichen Domäne des 5-LO-Enzyms angreift und stellt daher eine neue Leitstruktur für 5-LO-Inhibitoren dar, die chemopräventive Eigenschaften und ein verbessertes gastrointestinales Profil besitzen. Es lässt sich feststellen, dass die 5-LO-Hemmung durch Ssi einen neuen molekularen Ausgangspunkt für die Erklärung der COX-unabhängigen pharmakologischen Effekten von Ssi in der Therapie bietet.

Im dritten Teil der Arbeit wurde ein möglicher Einfluss von Ssi auf die 5-LO Genexpression untersucht. Es konnte im quantitativen PCR Versuch eine Regulation von Ssi auf die 5-LO mRNA-Menge in MM6-Zellen beobachtet werden. Der Effekt trat in differenzierten Monozyten (Differenzierungsdauer 72h mit Calcitriol und transforming-growth-factor-ß (TGF-ß) nach 72h Behandlung mit Ssi auf. Die 5-LO mRNA-Menge wurde in differenzierten MM6-Zellen auf ~20% der Kontrolle herunterreguliert. Da in der Literatur die Beteiligung des Transkriptionsfaktors Sp1 bei der Herabregulation der COX-Genexpression durch NSAR publiziert wurde, lag die Untersuchung der Sp1 Expression nahe. Simultan zur Abnahme der 5-LO mRNA Menge ließ sich eine Abnahme der Sp1 mRNA Menge feststellen. Daher sollte anschließend festge-

stellt werden, ob sich der durch Ssi-vermittelte Effekt auf die 5-LO-mRNA durch Überexpression von Sp1 supprimieren lässt. In der Reportergenanalyse konnte gezeigt werden, dass Sp1 bei der Ssi-vermittelte Herabregulation der 5-LO mRNA eine Rolle zu spielen scheint, da steigende Mengen an Sp1 den durch Ssi hervorgerufenen Effekt teilweise aufheben konnten. Ein Chromatin-Immunopräzipitationsversuch (ChIP-Versuch) lieferte dann bestätigende Ergebnisse, da in Anwesenheit von Ssi in der höchsten eingesetzten Konzentration von 30µM nahezu keine Bindung mehr von Sp1 an 5-LO detektierbar war. Ssi zeigt offenbar auch auf der Ebene der Genexpression regulatorische Effekte am 5-LO Enzym.

Als letztes beschäftigte sich die vorliegende Arbeit mit der Testung von NO-NSAR (NO-Sulindac, NO-Naproxen, NO-Aspirin) im Hinblick auf eine eventuelle 5-LO-Hemmung, da die chemopräventiven Wirkungen dieser Verbindungen möglicherweise auch über eine Hemmung des 5-LO-Proteins erklärbar sind. In intakten Granulozyten zeigten alle Verbindungen effektive Inhibition der LT-Biosynthese wobei NO-Sulindac am potentesten agierte (IC_{50} ~3µM). Die entsprechenden analogen Verbindungen ohne NO-Gruppe wurden als Kontrollsubstanzen ebenfalls getestet und hatten keinen Einfluss auf die 5-LO-Aktivität. NO-Aspirin hemmte mit einem IC_{50}-Wert von ~30µM. Weiterhin wurde das Verhalten von NO-Asprin in Anwesenheit und Abwesenheit des natürlichen Substrates AA untersucht. Die resultierenden ähnlichen Dosis-Wirkungskurven mit oder ohne AA ließen auf ein nicht-kompetitives Verhalten von NO-Aspirin im zellulären Testsystem schliessen. Versuche mit rekombinantem, aufgereinigtem 5-LO-Enzym ergaben wiederum eine Hemmung der 5-LO-Produktbildung durch alle drei Verbindungen. NO-Sulindac unterdrückte am potentesten die 5-LO-Produktbildung. Daraufhin wurden Tests im humanen Vollblutsystem durchgeführt. Es zeigte sich in diesem Testsystem jedoch eine deutlich schlechtere Inhibition bezüglich der Produktbildung von Leukotrien B_4 (LTB_4) und 5-Hydroxyeicosatetraensäure (5-HETE) durch NO-NSAR. Es waren höhere Konzentration der Substanzen im Vergleich zu den vorherigen Experimenten nötig (100µM), um mindestens 55% Enzymhemmung hervorzurufen. Die Strategie der 5-LO-Hemmung durch NO-NSAR ist von pharmakologischer Relevanz und es bedarf weiterer Untersuchungen, um den genauen molekularen Mechanismus der Hemmung der 5-LO durch NO-NSAR aufzuklären.

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