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# Chapter 1

## Introduction and Scope of the Thesis

# Development of Novel Molecules to Study Lipoxygenase Activity in Its Cellular Context

## 1. Introduction

Inflammatory diseases include a vast array of disorders that, unfortunately, afflict millions of people worldwide. The therapeutic possibilities for many patients are still limited. In particular, the precise causes of the inflammatory diseases are often unknown and many patients do not respond to the current therapy.[1] In order to address this unmet clinical need, it is important to gain more insight in molecular mechanisms that drive inflammation. Notably, lipoxygenases (LOXs) and their metabolites play key regulatory roles in numerous chronic inflammatory diseases such as asthma, rheumatoid arthritis, inflammatory bowel disease, psoriasis, dermatitis and nephritis.[2–3] Therefore, this group of enzymes has been recognized as potential therapeutic targets.

The metabolism of arachidonic acid (AA), linoleic acid (LA) and other related polyunsaturated fatty acids (PUFAs) provides various families of lipid mediators that are involved in regulation of diverse physiological processes such as inflammation, cancer, reproduction and host defense.[2–3] These bioactive signaling products derived from PUFAs, including AA and LA, are collectively known as eicosanoids.[4–5] Aberrations in the formation of eicosanoids are involved in numerous diseases, such as asthma,[6] COPD,[7] atherogenesis,[2] diabetes,[8–9] stroke,[10] Alzheimer's disease[11] and Parkinson's disease,[12] as well as cancer.[13–14] The discovery of aspirin (COX-1 and -2 inhibitor), with an estimated 40,000 tons consumption each year[15], marked a great success in targeting lipid oxidizing enzymes. Currently, aspirin is widely used in various diseases, such as pain, fever, headache or other inflammatory conditions. Notably, the function and regulation of eicosanoids in related diseases is increasingly gaining attention in drug discovery programs. In 1996, the drugability of LOX enzymes was demonstrated by the approval of the orally active 5-LOX inhibitor, Zileuton, which was used for treatment of asthma until 2008.[16] Further exploration of the LOX enzyme class is needed to find novel inhibitors with improved metabolic stability and isoenzyme selectivity.

In this thesis, we will focus on the lipoxygenases (LOXs), especially 15-lipoxygenase-1 (15-LOX-1). We will introduce the family of LOX enzymes and their role in oxygenation of PUFAs to provide eicosanoids.[2,17] Subsequently, the current knowledge of the physiological and pathological roles of LOXs will be summarized. Finally, the molecular approaches to target LOX in drug discovery projects will be discussed.

## 2. Lipoxygenase

LOXs are nonheme iron-containing enzymes, which are found widely in plants, fungi, and animals.[18] Furthermore, the family of mammalian LOXs are classified according to their positional and regiospecific peroxidation of AA. The regiospecificity of human LOX enzymes for AA peroxidation is used for subclassification into 5-LOXs, 8-LOXs, 12-LOXs or 15-LOXs (Figure 1).[18] In particular, there are two 15-LOXs isoenzymes that can be classified further as 15-LOX-1 and 15-LOX-2. 15-LOX-1 is the reticulocyte/leukocyte type and 15-LOX-2 is the epidermis type.[19] The amino acid sequences of 15-LOX-1 and 15-LOX-2 share 40% similarity. Moreover, 15-LOX-1 demonstrates a close sequence homology to 12-LOX (65%). In contrast to 15-LOX-1, 15-LOX-2 contains an in-frame 87-bp deletion.[20] On the one hand, the similarities of the sequence homology in these two isozymes can be found in highly conserved regions, such as the active site ligand for the iron atom. On the other hand, the 60% difference of non-identical amino acid sequence gives these two enzymes different affinities to their substrates. To be exact, 15-LOX-1 dioxygenates both LA and AA with comparable rates, and the dioxygenation of AA occurs at both C15 and C12 with a ratio of about 12:1. In contrast, 15-LOX-2 shows an exclusive preference to AA.[21] In addition, 15-LOX-1 and 15-LOX-2 have also been identified by their differences in tissue distribution. 15-LOX-1 is highly expressed in leukocytes and airway endothelial cells. In contrast, 15-LOX-2 is expressed in prostate, lung, cornea, and many other tissues such as liver, colon, kidney, spleen, ovary, and brain, but not in leukocytes.[22] Moreover, cells induced by interleukin (IL)-4 and IL-13 show a selective increase of 15-LOX-1 expression and not 15-LOX-2 expression, which is an important difference between 15-LOX-1 and 15-LOX-2.[21]

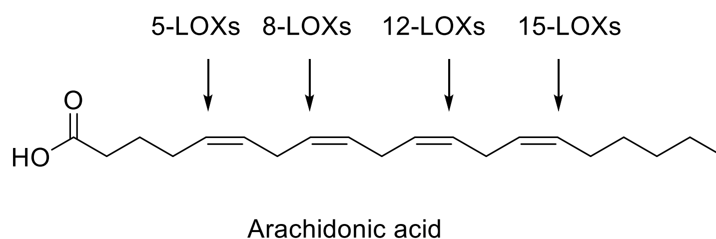


**Figure 1.** The nomenclature of the family of LOXs is based on their positional and regiospecific introduction of the molecular oxygen in the fatty chain of arachidonic acid (AA). More specifically, AA oxygenation can take place either at positions C-5 (5-LOXs), C-8 (8-LOXs), C-12 (12-LOXs) or C-15 (15-LOXs).

## 2.1 Free radical mechanism of lipoxygenases

LOXs are a group of nonheme iron-containing dioxygenases that catalyze a free radical reaction by which molecular oxygen is inserted into PUFAs with one or more *cis*,*cis*-1,4-pentadiene moieties. In this part, we take the classic catalytic mechanism of 15-LOXs as an example (Figure 2).[23–24] In general, the catalytic reaction consists of four steps, hydrogen abstraction, rearrangement, oxygen insertion and radical reduction. The $Fe^{3+}$ containing active site of activated 15-LOXs causes a single electron oxidation of arachidonic acid or linoleic acid that is bound to the active site. This results in a carbon centered radical that combines with $O_2$, which generates a new radical. The radical endoperoxide oxidizes the $Fe^{2+}$ to $Fe^{3+}$ for the next catalytic cycle.

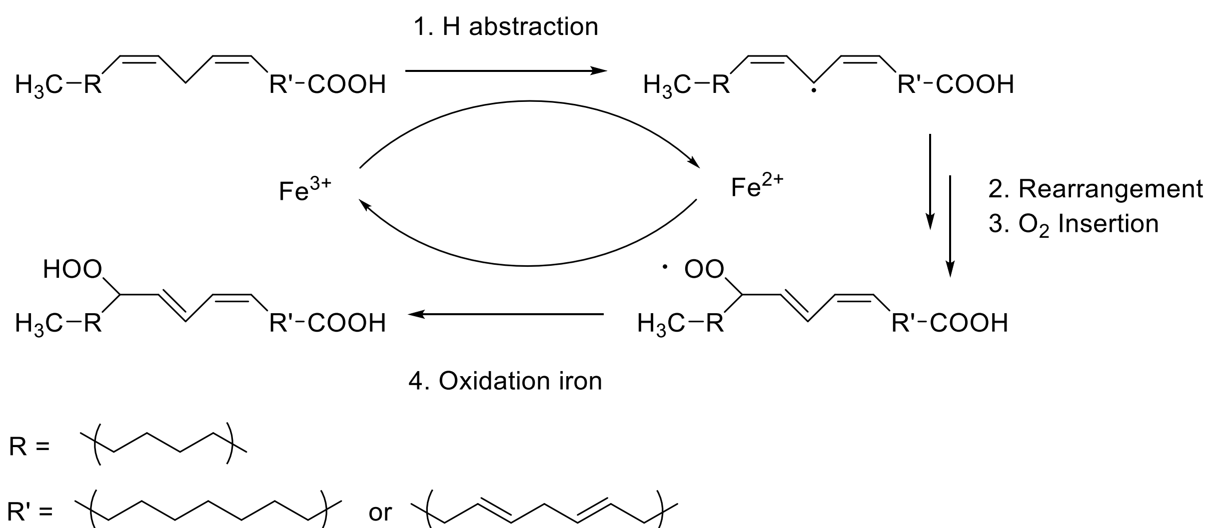


**Figure 2.** Catalytic cycle for the conversion of arachidonic acid (AA) or linoleic acid (LA) by 15-LOXs activity. Iron (III) causes single electron oxidation of arachidonic acid or lineoleic acid and converts into iron (II). This results in a carbon centered radical that combines with $O_2$, which generates a new radical. The radical allylperoxide oxidizes the iron (II) to iron (III) for the next catalytic cycle.

## 2.2 Substrates and products of 15-LOXs

15-LOXs are involved in the synthesis pathways of many important biologically active products, such as leukotrienes and lipoxins.[25] These compounds belong to the eicosanoids, which form a group of versatile signaling molecules.[13] One of the most important substrates of 15-LOXs is AA. This PUFA is a precursor for many biologically active molecules. As described earlier, the nomenclature of the LOX family is based on the position of the carbon at which the enzyme selectively introduces the hydroperoxide (Figure 3).[17,19] However, 15-LOX-1 is known to convert AA into 12- and 15-hydroperoxyeicosa-tetraenoic acids (HpETEs). The ability of 15-LOXs to produce both 12- and 15-HpETE shows that the selectivity of 15-LOXs are not completely at C15, because 15-LOXs also have some activity at C12.[17] The formed hydroxperoxides (HpETEs) by 15-LOXs are rapidly converted to 12- and 15-hydroxyeicosatetraenoic acids (HETEs). To summarize, 15-LOXs can oxidize AA at both C12 and C15, forming four different products, 12(*S*)-HpETE, 15(*S*)-HpETE, 12(*S*)-HETE and 15(*S*)-HETE.

Another important substrate for 15-LOXs is LA (Figure 3). The fatty chain of LA only contains one *cis*, *cis*-pentadiene moiety. 15-LOXs converts LA into 13-hydroperoxyoctadecadienoic acid (13(*S*)-HpODE), which can be further reduced to the respective hydroxy fatty acids, 13-hydroxyoctadecadienoic acid (13(*S*)-HODE). Certainly,

both of them are also important biologically active compounds that both activate and inhibit the regulation of proliferator-activating receptor-γ (PPAR γ).[19]

Some 15-LOXs products, such as 12(*S*)-HETE, 13(*S*)-HODE and 15(*S*)-HpETE act pro-inflammatory. The pro-inflammatory effect of 15(*S*)-HpETE and 13(*S*)-HODE includes the stimulation of protein kinase C (PKC) and translocation of Ras.[25–26] Furthermore, 15(*S*)-HpETE and 12(*S*)-HETE stimulate several cellular adhesion molecules, ICAM-1, ELAM-1, and VCAM-1, which leads to the binding of monocytes to blood vessel walls.[17] Another pro-inflammatory product of LA is 13(*S*)-HpODE, which stimulates the pro-inflammatory transcription factor NF-κB.[27] The production of the pro-inflammatory products explain why 15-LOXs are linked with the progression of inflammatory diseases.

15-LOXs are also involved in the anabolic pathway towards formation of resolvins and lipoxins and protectins, which exhibit important biological functions. In contrast to the pro-inflammatory products such as HETEs, HpETEs and HODEs, the lipoxins, resolvins and protectins are products of 15-LOXs with anti-inflammatory properties.[25,28] The name of lipoxins originates from lipoxygenase interaction products. Lipoxins are synthesized from AA via a pathway involving 5, 12, and 15-LOXs. The lipoxins stimulate resolution of inflammation, inhibit the function of neutrophils and increase vasodilation.[19] Resolvins are another class of anti-inflammatory products of 15-LOXs. They are synthesized by 15-LOXs from *omega*-3 PUFAs, such as docosahexanoic acid. The resolvins that are produced by 15-LOXs are called D-series (docosahexanoic) resolvins. Besides D-series resolvins, 15-LOXs are also able to convert docosahexanoic acid into protectins.[29–30] This third type of anti-inflammatory products of 15-LOX activity has been shown to be important in respiratory diseases, such as asthma, and is expected to prevent renal damage after ischemic renal injuries.

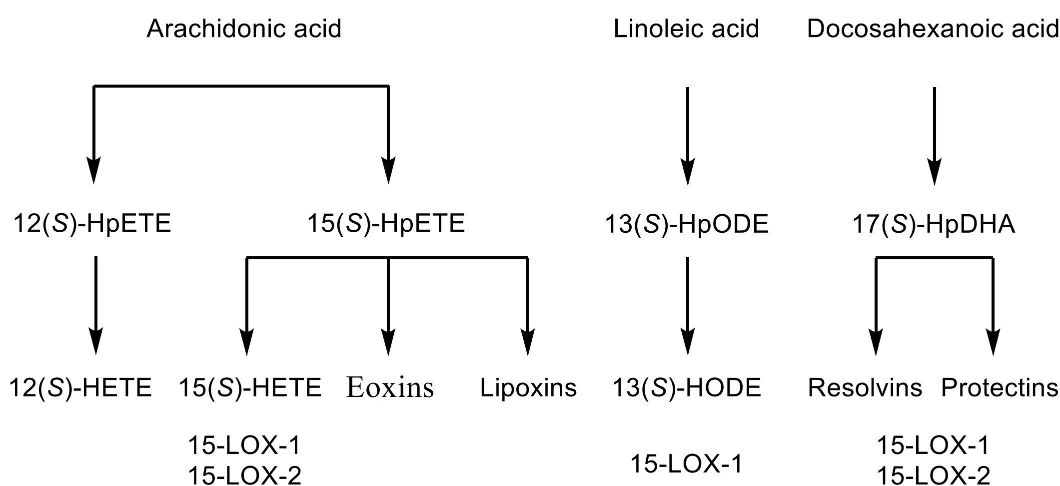


**Figure 3**. The action of 15-lipoxygenase enzymes on its lipid substrates and the formation of corresponding groups of products. When arachidonic acid is metabolized, all of the different 15-LOX isoforms generate lipid hydroperoxides (HpETEs) as the primary products. The latter are rapidly reduced intracellularly to their corresponding hydroxides (HETEs). Another category of metabolites that is generated by the sequential action of 15-LOX are the lipoxins and eoxins. 15-LOX-1 can also metabolize linoleic acid to generate 13(*S*)-HpODE (hydroperoxyoctadecadienoic acid) which is further peroxidized to 13(*S*)-HODE. Docosahexanoic acid is also a substrate for 15-LOX-1 that is metabolized by 15-LOX into a hydroperoxy derivative, which is rapidly transformed into resolvins and protectins.

## 2.3 Physiological and pathological role of 15-LOX-1

Since some products of 15-LOXs catalyzed reactions act pro-inflammatory, while other products are anti-inflammatory, it is clear that the role of 15-LOXs in inflammation is complex.[25] Importantly, 15-LOX-1 plays an important role in physiology. In this case, lipid oxidation products of 15-LOX-1, such as lipoxins, can orchestrate the ordered course of the inflammatory process which further contribute to the blockade of acute inflammation, monocyte recruitment, sorting of apoptotic cells and resolution of inflammation.[28]

Changes in the activity or expression of 15-LOX-1 are associated with a large number of inflammatory diseases. Atherosclerosis is one of the diseases that is associated with altered 15-LOX-1 activity. 15-LOX-1 acts pro-inflammatory in this disease by oxidizing low-density lipoproteins (LDL), which stimulate plaque formation. Furthermore, the products of 15-LOX-1 initiate upregulation of adhesion-molecules and stimulate smooth muscle cell remodeling. Both of these events stimulate the progression of atherosclerosis.[31–32]

Inflammatory lung diseases such as asthma and COPD are another group of diseases that are associated with altered 15-LOX-1 activity.[28] 12(*S*)-HETE is capable of increasing

vascular permeability, which stimulates the progression of inflammation. Furthermore, a study was performed in which 15-LOX-1 was overexpressed in mice with IL-13. The overexpression led to an increase of the metabolites of 15-LOX-1. Symptoms of asthma were more severe after IL-13 induced 15-LOX-1 upregulation due to an excess of metabolites. Furthermore, epithelial damage and the number of apoptotic cells were significantly higher than in 15-LOX-1 deficient mice that were also treated with IL-13.[33] It is clear that altered 15-LOX-1 activity and an excessive supply of substrates can ultimately result in inflammatory diseases.

Apart from atherosclerosis and inflammatory lung diseases, altered 15-LOX-1 activity and disordered supply of 15-LOX-1 derived products are associated with many other diseases such as allergies, osteoporosis, hypertension, Alzheimer's disease, congestive heart failure, diabetes, Parkinson's disease and several forms of cancer.[2,28,34] Because patients do not always respond to the current therapy novel therapeutics need to be developed. To address this unmet clinical need, it is important to gain more insight in molecular mechanisms in which LOXs are involved. In this thesis we focus on 15-LOX-1.

## 3. Development of chemical tools to investigate LOXs

The role of 15-LOXs in diverse diseases has triggered interest in the development of 15-LOXs inhibitors for drug discovery (Figure 4). Importantly, Zileuton was already proven to be successful as an orally active inhibitor of 5-LOX for the maintenance treatment of asthma by inhibition leukotriene formation (LTB4, LTC4, LTD4, and LTE4).[16] This development demonstrates that the LOXs family enzymes are a drugable class or enzymes. In contrast to Zileuton, none of the known 15-LOX inhibitors has reached clinical trials, because of limited potency or unfavorable physical chemical properties. Therefore, more inhibitors with new chemotypes and improved physical-chemical properties are needed to explore the utility of 15-LOX-1 as a novel drug target. **PD-146176** is a frequently used competitive and selective 15-LOX-1 inhibitor. This inhibitor has an $IC_{50}$ value of 3.81 μM and shows no effect on 5-LOX, 12-LOX, COX-1 or COX-2.[35] This discovery stimulated more efforts to develop 15-LOX-1 inhibitors with an indolyl core. More researchers reported the discovery of indole-based or indole-like 15-LOX-1 inhibitors, **371** and **4b** (with $IC_{50}$ of 0.006 and 3.84 μM, respectively).[36–37] Furthermore, 1,3-oxazole based compound (**ML351**) was identified as 15-LOX-1 inhibitor.[10] Moreover, also non-specific LOX inhibitors, such as Baicalein, were also identified. Baicalein has since been shown to inhibit both 12-LOX and 15-LOX ($IC_{50}$ =

0.64μM for 12-LOX and 1.6μM for 15-LOX-1).[38] In our group, we previously discovered the 15-LOX-1 inhibitor, Eleftheriadis-**14d**, which also contains an indole core and demonstrates a good potency ($IC_{50}$ = 90 nM).[39] These findings set the stage for further exploration of the substitution around the indole core in order to optimize both 15-LOX-1 inhibition and physical-chemical properties for drug discovery.

Complementary to development of inhibitors, efforts were made to engineer 15-LOX-1 substrates for detection of enzyme activity that can help understanding of the functional behavior of proteins in inflammatory diseases. Previously, we developed an activity-based probe **N144** as a chemical reporter for lipoxygenase activity in cell lysates and tissue samples.[40] Probe **N144**, mimicking the natural substrate of 15-LOX-1, is proposed to covalently bind with the active site of 15-LOX-1 to provide a mechanistic basis for activity-based labeling of 15-LOX-1. This two-step labeling on 15-LOX-1 is performed by incubation of probe **N144** with cell lysates, followed by biotinylation via the oxidative Heck reaction overnight. Another study employed the *omega*-alkynyl fatty acid (**aAA**) to identify the intracellular targets of 12/15-LOX-generated lipid-derived electrophiles.[41] This sets the stage for the development of potent 15-LOX-1 inhibitors and to study their cellular activity. In **chapter 4** we describe the development of probes for activity-based labeling in one step. Application of these probes will expand our understanding of the functional behavior of LOXs enzymes in their cellular context. In particular, the combination of small molecule inhibitors and advanced chemistry-based methods for detection of LOX enzyme activity will advance our understanding of the roles of this class of enzymes in their physiological context.

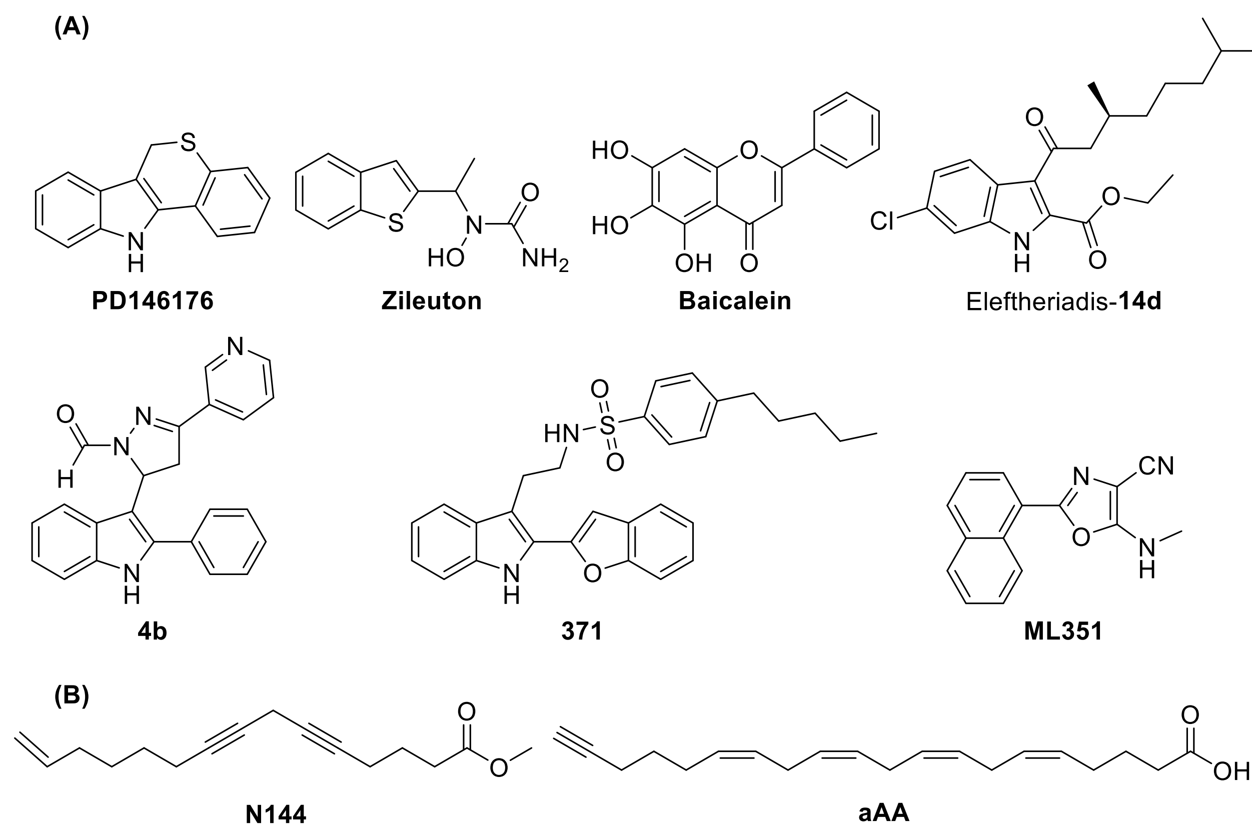


**Figure 4**. Examples of previously reported LOX (lipoxygenase) inhibitors and chemical tools to study lipoxygenase activity. (A) Previously reported LOX inhibitors. (B) Substrate-based chemical tools to study lipoxygenase activity in cell-based systems.

## 4. Scope of the thesis

15-lipoxygenase-1 (15-LOX-1) is an enzyme involved in the biosynthesis of inflammatory signaling molecules having key regulatory roles in immune responses and numerous diseases, such as asthma, COPD, atherogenesis, diabetes, stroke, Alzheimer's disease and Parkinson's disease, as well as cancer. For millions of patients suffering from these diseases, therapeutic possibilities that can address these unmet clinical needs are urgently needed. Therefore, we aim to develop small molecular inhibitors and chemistry-based detection methods for LOX enzymes in order to gain more knowledge and expand the therapeutic possibilities for these inflammatory diseases. In **Part 1** including **chapter 2** and **3**, we present the development of novel 15-LOX-1 inhibitors and their biological evaluation in cell-based studies. In **Part 2** including **chapter 4**, we present the development of novel 15-LOX-1 inhibitors in combination with the development of novel probes for activity-based detection of 15-LOX-1 activity.

### 4.1. Part 1 – Development of novel 15-LOX-1 inhibitor

In **chapter 2**, the 2-aminopyrrole scaffold was selected as a starting point for identification of novel h-15-LOX-1 inhibitors using substitution-oriented screening (SOS) of about 200 2-aminopyrrole inhibitors. The novel inhibitor **21B10** ($IC_{50}$ = 11.8 ± 2.3 μM) was explored as the initial hit and another 29 compounds were successfully synthesized using multi-component reaction (MCR) chemistry in order to gain understanding of their structure–activity relationships (SAR). The $IC_{50}$ for the most potent inhibitor of this series is 6.3 μM and the enzyme kinetics demonstrated uncompetitive inhibition. Furthermore, we found that the viability of HCC-1.2 cells was inhibited. The similarity of **21B10** to benzophenone triggered us to investigate photoactivation as a plausible mechanism of inhibition for 15-LOX-1. Specifically, photoactivation can cause generation of free radicals, which triggers covalent binding of the molecule to amino acid residues in the enzyme active site. Indeed, we found photoactivation both at the enzymatic and cellular level after the exposure to UV-irradiation (365 nm) and visible light. This suggested that the 2-aminopyroles might act as pan assay interfering substances, presumably acting via a radical mechanism.

In **chapter 3**, we reported the synthesis of novel molecules and their inhibition of 15-LOX-1 activity. Structure-activity relationships for binding to 15-LOX-1 were investigated starting from the core scaffold ethyl 6-chloro-1*H*-indole-2-carboxylate ($IC_{50}$ = 3 μM) and the

more elaborated Eleftheriadis-**14d** ($IC_{50}$ = 0.09 μM). 24 molecules were successfully synthesized and tested for 15-LOX-1 inhibition, which provided insight into the structure-activity relationships. The best inhibitor **9c (i472)** of this series displays an $IC_{50}$ of 0.19 μM against 15-LOX-1. This new potent inhibitor has fewer rotatable bonds with a better cLogP value that is more favorable for cellular permeability compared to the previously identified inhibitor Eleftheriadis-**14d**. In this study, we also demonstrated that **9c (i472)** is an inhibitor of cellular lipoxygenase activity in RAW264.7 macrophages using activity-based labeling. Additionally, our results showed that **9c (i472)** protects RAW 264.7 macrophages from LPS-induced cell death and exhibits significantly better dose-dependent effects when compared to Eleftheriadis-**14d**. Furthermore, **9c (i472)** was shown to provide significant inhibition of NF-κB transcriptional activation upon LPS/INFγ stimulation, to downregulate the expression of the NF-κB related gene iNOS, to provide dose-dependent inhibition of NO production and to reduce lipid peroxidation in RAW macrophages. Importantly, this work provided a new and potent inhibitor of 15-LOX-1, and showed evidence that inhibition of 15-LOX-1 can downregulate the formation of oxidative mediators, such as lipid peroxides and NO.

## 4.2. Part 2 – The combination of inhibition and detection of 15-LOX-1

In **chapter 4**, we highlight the combination of chemistry-based inhibition and detection of LOX activity. We aimed to develop novel probes for convenient activity-based labeling of LOX enzymes and to employ them for screening of novel 15-LOX-1 inhibitors on the cellular level. We anticipated that combination of these techniques can push the drug discovery process forward.

Firstly, we designed and obtained a series of novel indole-based 15-LOX-1 inhibitors and the $IC_{50}$ value of the most potent inhibitor (**i472a**) is 20 nM. Next, we created a one-step activity-based labeling for 15-LOX-1 instead of a two-step labeling that we reported previously. For this novel 15-LOX-activity-based probe, the bis-alkyne functionality was maintained as core structure and a biotin was attached as a detection group. A series of novel 15-LOX-1 activity-based probes was synthesized and their SAR for 15-LOX-1 binding was investigated. The most potent covalent inhibitor provided an $IC_{50}$ value of 2.6 μM against 15-LOX-1. This molecule was used to evaluate a series of known and novel non-covalent 15-LOX-1 inhibitors for LOX inhibition in a cellular context. In sum, our results showed that the activity-based labeling has potential for the investigation of cellular lipoxygenase activity.

## 4.3. Part 3 – Summary and future perspectives

All results are summarized and discussed in **chapter 5** in which we also provide future perspectives.

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