Development of novel molecules to study lipoxygenase activity in its cellular context

Guo, Hao

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Chapter 5

Summary and Perspectives

Summary

Chronic inflammatory diseases, such as asthma and chronic obstructive pulmonary disease (COPD), afflict millions of people worldwide. Unfortunately, the therapeutic possibilities for many patients are limited, because they do not respond to the current therapy.[1–2] For these chronic inflammatory diseases, many therapeutics are partially effective or ineffective and cause many side effects.[3] On the other hand, increasing evidence shows that inflammation has a close link with cancer and disorders of the central nervous system (CNS).[4–5] Therefore, expansion of the therapeutic possibilities is highly needed. To address these unmet clinical needs, it is important to gain more insight in molecular mechanisms that drive inflammation.

Recent technological developments in molecular biology have led to a tremendous increase in our understanding of inflammation. Nevertheless, our understanding of the functional behavior of proteins in inflammatory diseases remains limited that the activity of their regulating enzymes is poorly studied. One of the most important strategies in pharmacology is the application of small molecule inhibitors as tools to investigate the role of enzyme activity in its physiological context. In addition, detection of alterations in enzyme activity in cell-cultures or tissue samples requires, advanced chemistry-based detection methods in some cases. Currently, the lack of chemistry-based tools to study inflammatory processes is a bottleneck for progress in our understanding. Therefore, we developed tools to modulate and detect enzyme activity in its cellular context, which will ultimately open up novel opportunities in drug discovery and diagnosis.

Our research focusses on the enzyme 15-lipoxygenase-1 (15-LOX-1). This is an enzyme that converts arachidonic acid and linoleic acid into eicosanoids, which are lipid signaling molecules that play versatile roles in the immune system. 15-LOX-1 catalyzes conversion of arachidonic acid and linoleic acid in 15(*S*)-HETE and 13(*S*)-HODE, respectively. These lipid-signaling molecules contribute to inflammatory processes in asthma.[6] Furthermore, the lipid peroxides produced by 15-LOX-1 catalysis play key roles in oxidative stress (**chapter 3**). In addition, pro-inflammatory mediators such as cytokines, prostaglandins and leukotrienes stimulated by metabolites of 15-LOX-1 show a close connection with CNS diseases like Alzheimer's, Parkinson's disease and stroke. As 15-LOX-1 plays a crucial role in the regulation of lipid-signaling molecules with many important

regulatory roles in various diseases, 15-LOX-1 appears as an emerging target in drug development.

Recently, 15-LOX-1 has gained more attention for the development of novel anti-inflammatory and analgesic drugs due to the success in drug development in similar pathways such as cyclooxygenase-2 (COX-2). Importantly, the expression of the enzyme, 15-LOX-1, is upregulated in inflamed tissue, which suggests a selective action of this enzyme in this context. The drugability of LOXs is demonstrated by the orally active 5-LOX inhibitor, Zileuton, which is used for treatment of asthma. There are indications that 15-LOX-1 could also be employed as a drug target in airway inflammation. However, very little selective, potent and cell-permeable small molecule inhibitors are available for 15-LOX-1. Interestingly, our group identified and developed a series of novel 15-LOX-1 inhibitors (**Part 1**) and activity-based probes (**Part 2**) that provide a good starting point for fundamental research as well as drug development projects.

Part 1: Inhibition of 15-LOX-1

In **chapter 2**, we aimed to identify novel inhibitors for human 15-lipoxygenase-1 (h-15-LOX-1), because of its potential as drug target in inflammation and cancer. We employed substitution-oriented screening (SOS) for the identification of inhibitors with novel substitution patterns. We identified novel inhibitors with a 2-aminopyrrole scaffold as inhibitors for h-15-LOX-1. The observed structure activity relationships (SAR) proved to be relatively flat. IC_{50}'s for the most potent inhibitor of the series did not surpass 6.3 µM and the enzyme kinetics demonstrated uncompetitive inhibition. Based on this, we hypothesized that the investigated 2-aminopyrroles are pan-assay interference compounds (PAINS) that act upon photoactivation via a radical mechanism.[7–8] Our results demonstrated a clear photoactivation of h-15-LOX-1 inhibition under UV and visible light. In addition, the investigated 2-aminopyrroles decreased the viability of cultured human hepatocarcinoma cells HCC-1.2 in a dose-dependent manner with LD_{50} ranging from 0.55 ± 0.15 µM (**21B10**) to 2.75 ± 0.91 µM (**22**). This verifies the role of inhibition of h-15-LOX-1 in cancer. Taken together, this indicates that photoactivation can play an important role in the biological activity of compounds with a 2-aminopyrrole scaffold as investigated here (Figure 1).

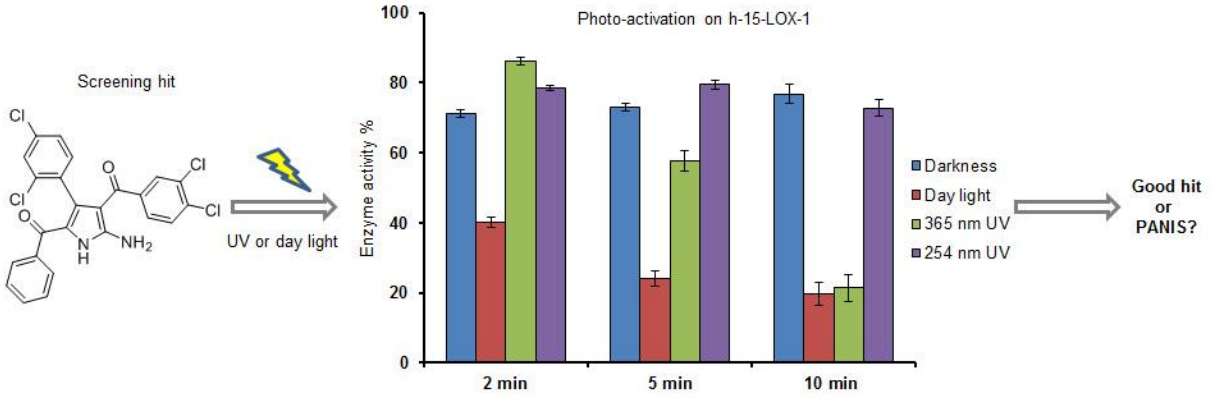

Figure 1. The outline of chapter 2. We used substitution-oriented screening (SOS) as a tool to the identify 2-aminopyrrole scaffold for inhibition of h-15-LOX-1. Next, we explored the function of photoactivation in the biological activity.

In **chapter 3**, we reported on the synthesis of novel inhibitors of 15-lipoxygenase-1 (15-LOX-1), an enzyme involved in the biosynthesis of inflammatory signaling molecules having key regulatory roles in immune responses and numerous diseases. It is widely accepted that various mechanisms for regulated cell death include the formation of oxidative mediators such as lipid peroxides and nitric oxide (NO).[9–10] In this respect, 15-LOX-1 is, by catalysis of lipid peroxidation, a key enzyme. The actions of these peroxides are interconnected with nuclear factor-κB (NF-κB) signaling and NO production. Inhibition of 15-LOX-1 holds chances to interfere with regulated cell death in inflammatory conditions.

In the work described in **chapter 3**, a novel potent 15-LOX-1 inhibitor, **9c (i472)**, was developed and structure–activity relationships were explored. We demonstrated that **9c (i472)** is an inhibitor of cellular lipoxygenase activity in RAW264.7 macrophages using activity-based labeling. Additionally, we successfully established that **9c (i472)** protects RAW 264.7 macrophages from LPS-induced cell death and showed significantly stronger 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 (Figure 2). Based on this study, we pave the way for the development of new inhibitors influencing different cell death mechanisms that might lead to new therapeutics to control diseases, such as acute lung injury and acute respiratory distress syndrome (ARDS).

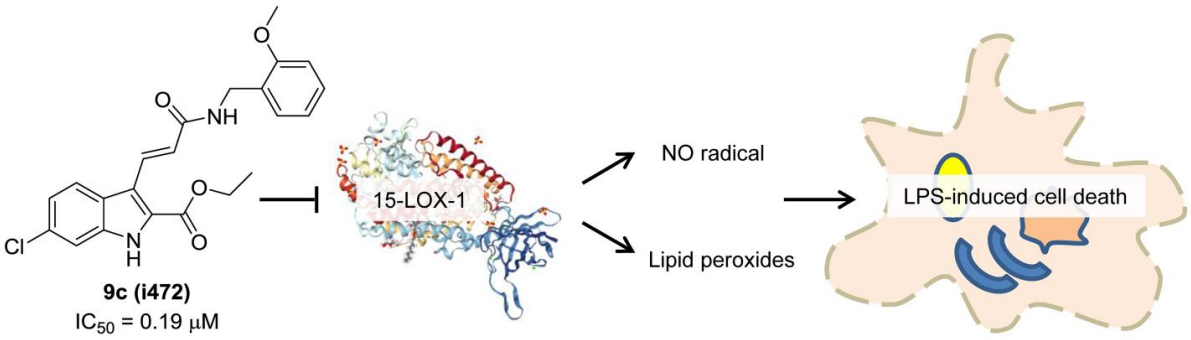

Figure 2. The outline of chapter 3. We explored a novel 15-LOX-1 inhibitor in a study for regulated cell death and inflammation.

Part 2: Detection of 15-LOX-1

In **chapter 4**, we combined the study of inhibition and detection. 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** proved to be 20 nM. Next, we created a probe for one step activity-based labeling of 15-LOX-1 to replace our previously developed two step labeling method. For this novel 15-LOX-activity-based probe, the bis-alkyne was kept as core structure and a biotin tail was included as a detection group. A series of novel 15-LOX-1 activity-based probes was synthesized and SAR studies were performed as described in **chapter 4** (Figure 3). In the end compound **17** (**D0**4), with an IC_{50} value of 2.6 μM against 15-LOX-1, proved to be an interesting molecule for activity-based labeling. This molecule was applied for activity-based labeling performed in living cells followed by cell lysis and on blot detection. This activity-based labeling was used to compare the potency of a series on inhibitors in its cellular context. With this development, we aim to enable monitoring inhibition of LOX enzymes in their cellular context, which will provide opportunities for drug discovery and development.

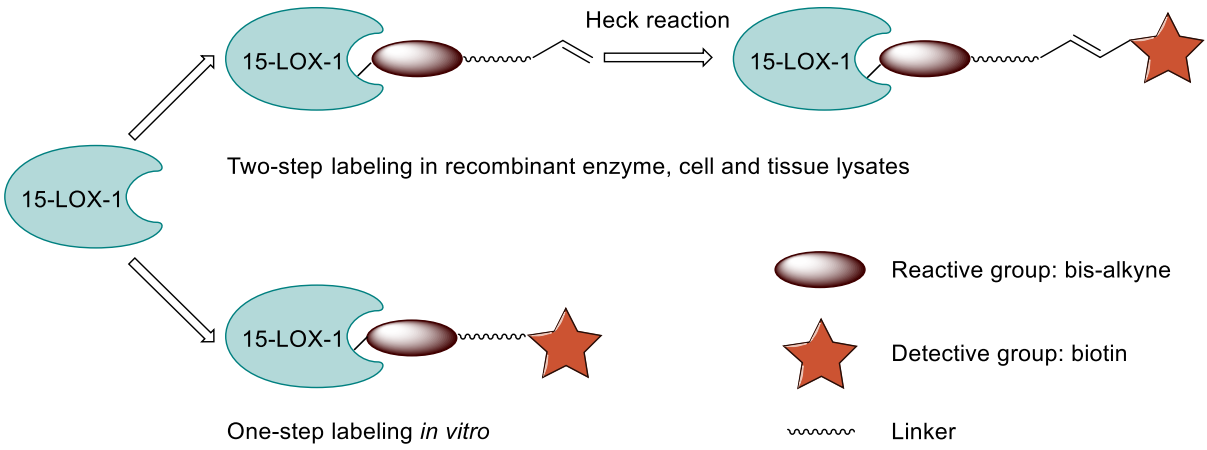

Figure 3. The development of 15-LOX activity labeling. For two-step labeling, isolated proteins or cell lysates are incubated with an activity-based probe for two minutes which is followed by the Heck reaction overnight. For the one step labeling the reactive group is already equipped with a detectable biotin group. This probe is incubated with the cell culture for two hours and the labeling is subsequently visualized on western blot.

Future perspective

Our studies on 15-LOX-1 during this PhD provided interesting information on this target. Our studies included multiple aspects, such as mechanism of inhibition, activity-based labeling and cellular activity. Progress of this work will open up novel opportunities in drug discovery and diagnosis.

As we described in **part 1**, we focus on the development of 15-LOX-1 inhibitors, that have potent inhibitory activity and physicochemical properties that are compatible or incompatible with cellular activity. In the structure−activity relationships (SARs) study, compound **i472a** proved to be the most potent 15-LOX-1 inhibitor with an IC_{50} value of 0.02 μM and a CLogP value of 5.2. However, with the results of molecular modeling and previously obtained SAR, it is to be expected that steric effects on the indole 3-position play key roles in the potency against 15-LOX-1. Furthermore, the substitution at the indole 2-position is also very important for inhibition of 15-LOX-1. Interestingly, molecular modeling shows that there is still space left to accommodate larger substitutions at the indole 2-postion. Hence, we anticipate it is promising to explore novel substitutions at both the 2- and 3-position of the indole scaffold (Figure 1). Ultimately, we hope that our efforts enable development of an active 15-LOX-1 inhibitor to expand the limited therapeutic options for inflammatory diseases.

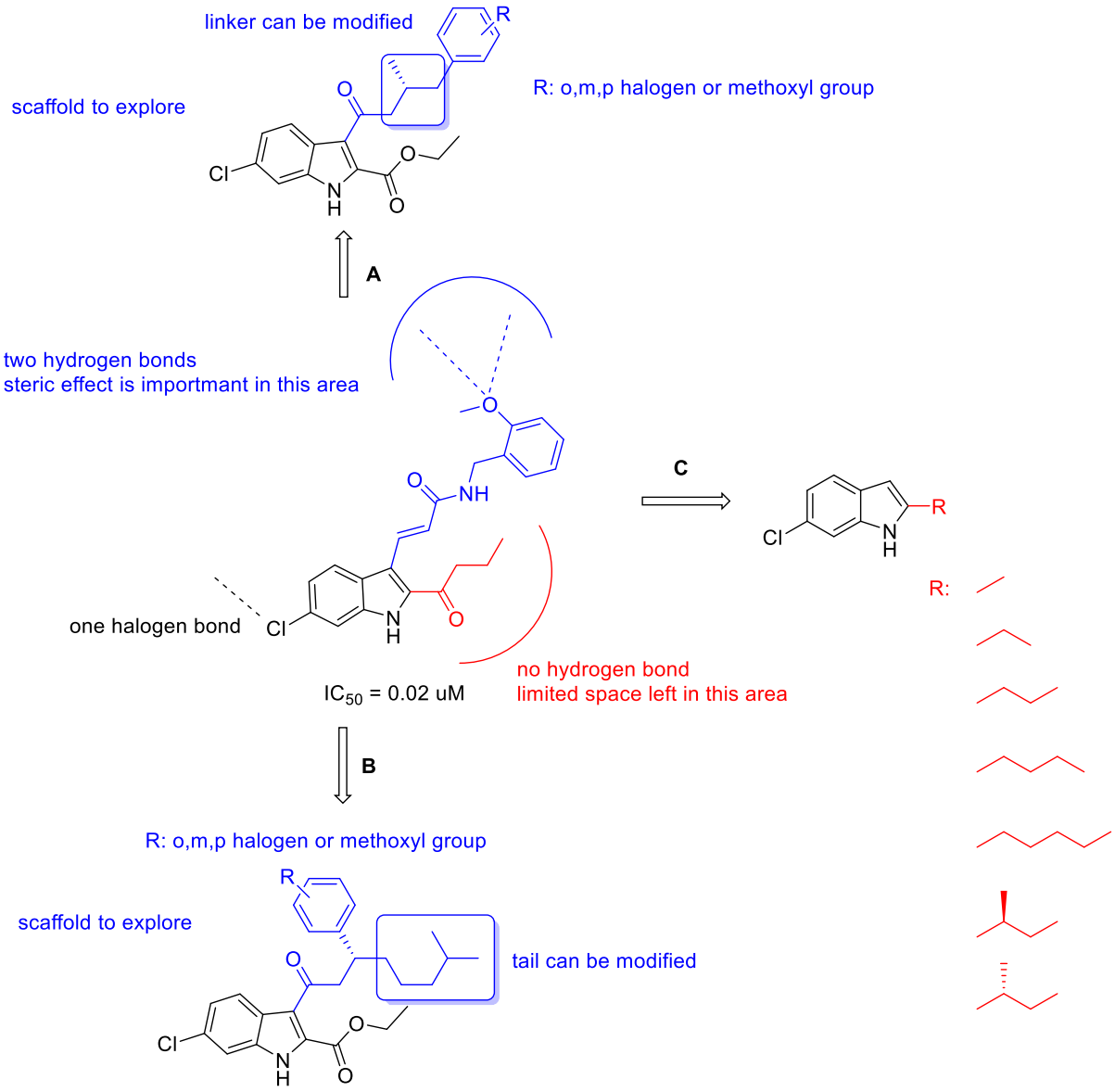

Figure 1. The outlook of development of 15-LOX-1 inhibitor. We anticipate to do more novel and steric modification at both of 2- and 3-position of indole. In the end, we will combine all the optimal modification from A, B and C in order to develop better 15-LOX-1 inhibitor.

In **part 2**, we reported a novel probe for one-step labeling of 15-LOX-1. The one-step labeling protocol is more convenient compared to the two-step labeling protocol. More importantly, we were able to apply the labeling in living cells to evaluate the LOX inhibitory potency of new and previously identified inhibitors.

In the future, we aim to implement more advanced studies on the LOX activity-based labeling. We aim to study the covalent labeling of 15-LOX-1 by mass spectrometry, but so far, we were not successful. In order to facilitate such studies we aim to design probes with better cleavable linkers compared to the ester in the currently applied probes. (Figure 2). In this way,

it should become more easy to remove non-labeled proteins and purify LOX enzymes from streptavidin agarose.

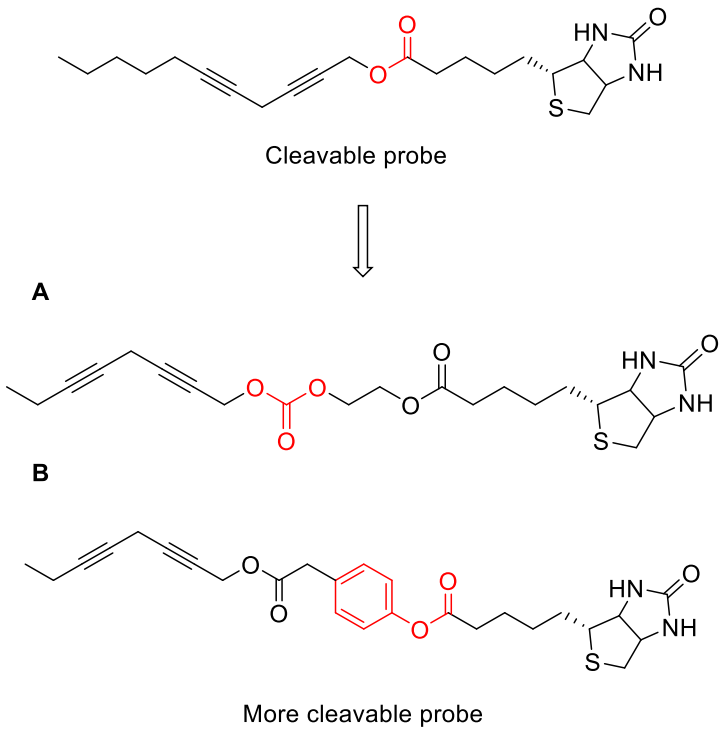

Figure 2. The development of 15-LOX-1 activity-based probe. Red region is the cleavable linker that can be easily remove in alkaline solution. (A) Designed cleavable probe with carbonate ester linker as leaving group. (B) Designed more cleavable probe with phenyl ester linker as leaving group.

Overall, our work shows a strong cross-talk mechanism in inflammation-related cell death. Most importantly, in contrast to inhibition of 5-LOX, inhibition of 15-LOX-1 downregulates the formation of lipid peroxides that might lead to new therapeutics to control diseases such as acute lung injury and acute distress syndrome. Furthermore, activity-based labeling creates and offers more possibilities to investigate enzyme activity in drug development. This advances drug discovery for enzymes with lipoxygenase activity and also provides indications on how to address oxidative enzyme targeting different groups of substrates. Finally, we anticipate that our efforts, ultimately contribute to creation of a new drug and a chemical diagnostic tools targeted at 15-LOX-1.

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