Inhibition and detection of 15-lipoxygenase-1

Eleftheriadis, Nikolaos

Summary

Inflammatory lung diseases like asthma and chronic obstructive pulmonary disease (COPD) can have detrimental effects on patients' health. Fortunately, nowadays these diseases can be alleviated by various therapeutic agents. Nevertheless, expansion of the therapeutic possibilities is needed, because for some patients the currently available medicines are ineffective or cause severe side effects. Therefore, the development of novel compounds targeting enzymes that are involved in inflammatory lung diseases is highly important.

Moreover, recent evidence shows that inflammation plays a major role in central nervous system (CNS) disorders. In various acute, chronic and psychiatric CNS disorders, pro-inflammatory mediators like cytokines, prostaglandins and leukotrienes, have been found to play pivotal roles. In addition, elevated levels of IL-1, IL-6 and TNFα have been identified in brain tissue of patients with Alzheimer's (AD) and Parkinson's (PD) disease. Furthermore, several animal studies suggest a connection between IL-1 and stroke, multiple sclerosis and depression. These findings have led to a search for novel therapeutic agents that can target inflammation in the CNS.

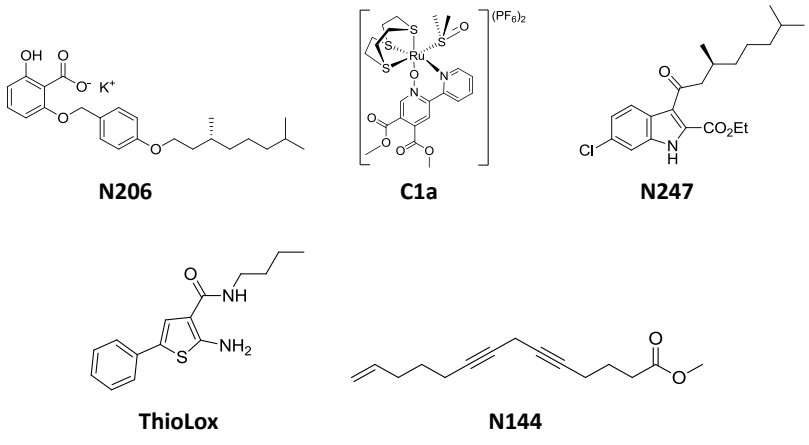
Human 15-lipoxygenase-1 (15-LOX-1) is an important mammalian lipoxygenase and plays a crucial role in the biosynthesis of inflammatory signaling molecules, having a regulatory role in several inflammatory lung diseases such as asthma, COPD and chronic bronchitis and more recently in various CNS diseases like Alzheimer's and Parkinson's as well as stroke. Novel potent inhibitors and activity-based probes of 15-LOX-1 are urgently required to explore the role of this enzyme further and enable drug discovery efforts. There are two parts presenting in this thesis. **Part A** includes chapters 2, 3, 4 and 5 in which we present the design of new inhibitors while **Part B** includes chapter 6 in which we described the synthesis of activity-based probes of 15-LOX-1.

Part A – Inhibition of 15-LOX-1

In **chapter 2**, we described the synthesis and the characterization of 6-benzyloxysalicylates as a new class of inhibitors of h-15-LOX-1. Enzyme inhibition and kinetic studies as well as molecular modeling studies were performed in order to characterize a structurally novel inhibitor **37a** (N206), which proved to be a competitive inhibitor of h-15-LOX-1 with a K_i value of 1.7 μM. Compound **37a** is the R enantiomer of the racemic mixture **8a**, while the S enantiomer, compound **37b** is 6-fold less active. Molecular modelling studies indicate that compound **37a** occupies most of the available space in the substrate binding pocket while the S-enantiomer **37b** demonstrates a mismatch of the aliphatic tail with the substrate binding pocket. These structure activity relationships provide a basis to design improved inhibitors and to explore 15-LOX-1 as a drug target.

Several types of ruthenium(II) (Ru(II)) complexes as novel inhibitors of 15-LOX-1 are presented in **chapter 3**. We present the first study that identifies Ru(II) complexes as novel inhibitors of 15-LOX-1. Two different types of complexes were tested with the general formulas: [Ru([9]aneS$_3$)(dmso)(*N,N*- or *N,O*-donor ligand)](PF$_6$)$_2$ and [(η^6-*p*-cymene)RuCl(*O,O*-ligand)]Cl. Among the seven tested Ru(II) complexes, two were newly synthesized (**C1a** and **C1b**). Both novel complexes were completely characterized and also their crystal structures have been determined. The data reported herein reveal four Ru(II) complexes as inhibitors of 15-LOX-1 with a potency in low micromolar range, whereas the respective free ligands showed no inhibition of the enzyme. Enzyme kinetic analysis of a Ru(II) complex (**C1a**) showed uncompetitive inhibition, which indicates that it binds to the substrate bound enzyme. In this study, we identify lipoxygenases as a new class of enzymes that is inhibited by Ru(II) complexes, which is important for a better understanding of the action of ruthenium based drugs.

In **chapter 4**, we employed an efficient strategy to identify structurally new inhibitors for the enzyme 15-LOX-1, which is an emerging drug target in various inflammatory diseases. Our approach started with a substitution oriented fragment screening (SOS) of a focused fragment library containing diversely substituted heterocycles. After the discovery of four hits, two inhibitors were selected to derive structure activity relationships and subjected to enzyme kinetic analysis, which indicated competitive inhibition. These observations were applied to support a molecular modeling study proposing a binding configuration in the active site of the enzyme h-15-LOX-1. Based on this model the substitution of the inhibitor was further optimized using structure-based design to provide inhibitor **N247** with a K_i of 36 nM and good ligand efficiency metrics. This inhibitor was evaluated in cell-based studies using RAW 264.7 macrophages and *ex vivo* studies in mouse precision-cut lung slices. The inhibitor provided an increase in the expression of IL-10 both in macrophages and mouse lung tissue.



N206 **C1a** **N247**

ThioLox **N144**

Figure 1. 15-LOX-1 inhibitors that have been presented in this thesis.

Based on the substitution oriented screening (SOS) approach, we designed inhibitors to explore 15-LOX-1 as a drug target in various inflammatory and neurological diseases, which is described in **chapter 5**. In this study, we utilized the SOS approach with MCR (Multicomponent Reactions) chemistry to identify the thiophene-based 15-LOX-1 inhibitor, **ThioLox**. The enzyme kinetic analysis as well as the molecular modeling studies showed competitive inhibition. **ThioLox** was calculated to have a K_i value of 3.30 µM, very good ligand efficiency metric but also the desired physicochemical properties. This inhibitor was evaluated in *ex vivo* studies in precision-cut lung slices (PCLS) of mouse lung tissue showing a strong anti-inflammatory effect. In addition, considering the acceptable physiochemical properties, neuroprotective studies were performed in HT-22 neuronal cells showing strong protection. Identification of **ThioLox** provides a starting point to target 15-LOX-1 with a competitive inhibitor with limited size and lipophilicity.

Part B – Detection of 15-LOX-1

A study of activity-based labeling of 15-LOX-1 is described in **chapter 6**. We have created for the first time an activity-based probe as an efficient chemical tool for activity-based labeling of recombinant 15-LOX-1 that also provides 15-LOX-1 dependent labeling in cell lysates and tissue samples. Towards this aim irreversible inhibitors for the target enzyme were designed and synthesized. An enzyme kinetic study of the novel inhibitors enabled the estimation of the potency along with the inactivation parameters and the inhibition mechanism. Subsequently, an alkene tag was introduced as a tag to enable biotinylation using the oxidative Heck reaction. Application of the alkene as a tag was needed to enable straightforward synthesis of the bis-alkyne probes. Here, we applied the oxidative Heck reaction for the first time for detection of activity-based labeled proteins thereby demonstrating the potential of this recently developed bioorthogonal coupling reaction in this type of applications. Activity-based labeling studies were performed on the recombinant enzyme, cell and tissue lysates. In all cases we demonstrated labeling of enzymes that could be attributed to 15-LOX-1 activity by application of heat inactivation and/or pharmacological inhibition.

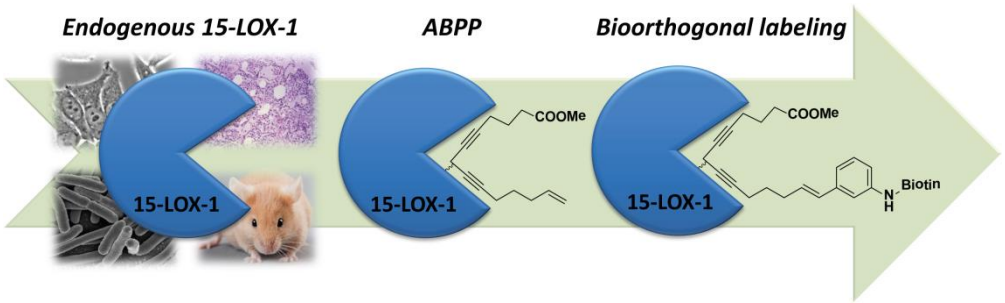


Figure 2. Two step identification of 15-LOX-1 using activity-based probe.

Future perspectives

As we briefly described, 15-LOX-1 appears as an emerging drug target for the treatment of inflammatory and neurological diseases. The work reported in this thesis has focused on the design of new inhibitors and activity-based probes for the enzyme 15-LOX-1.

Many of the known 15-LOX-1 inhibitors suffer from low inhibitory potency and/or poor physicochemical properties, such as a high logP value, which limits their potential therapeutic value. We developed an approach for screening of fragments that is focused on different scaffolds with a very diverse substitution pattern. This approach enables the identification of fragments, which can be further optimized by identification of Structure Activity Relationships (SAR) to support structure-based design. We denoted this approach Substitution Oriented fragment Screening (SOS). Using this approach, we identified inhibitors from different classes of compounds such as indoles and thiophenes. Our developed inhibitors have improved physicochemical properties as well as inhibitory potency compared to the previously described inhibitors. We reported a dual role of 15-LOX-1 and we used our new developed inhibitors to explore it. These drug-like inhibitors can be potentially used in more complex biological systems in order to define the role of 15-LOX-1. We anticipate that SOS approach on heterocycles can enable the exploration of very compact molecules with the desired physicochemical properties. For the next studies, the metabolic stability of the new inhibitors should also be taken in account. Therefore, the next SOS fragment library can be more focused on both very compact and metabolic stable molecules. This will lead to drug-like compounds that can be used to target the enzyme in *in vivo* studies.

Furthermore, we developed the first 15-LOX-1 activity-based probe as an efficient chemical tool for activity-based labeling of recombinant 15-LOX-1 that also provides 15-LOX-1 dependent labeling in cell lysates and tissue samples. The labeling of 15-LOX-1 was performed after incubation with the activity-based probe followed by biotinylation via oxidative Heck reaction. In the next studies, this two-step labeling can be modified to one-step labeling after using a modified probe with an attached biotin. One of the advantages of the one-step over two-step labeling is the faster experimental procedure. In comparison with our two-step labeling which describes a two days experimental procedure, the one-step labeling can be performed in one day. Moreover, our potent indole inhibitor **N247** can be converted to a activity-based probe after introducing the bis-alkyne functionality of the existing probe **N144**. The new indole probe could present a better selectivity profile because it is based on a very potent inhibitor which has a very high affinity for 15-LOX-1. Another idea is the use of a fluorophore instead of biotin for the labeling of the enzyme. This, would also make the experimental procedure faster while there is no need for Western blotting. Furthermore, different fluorophores present different detection limits, solubility and properties, that

can be chosen for diverse applications. We anticipate that further development of this type of molecules will enable the investigation and identification of 15-LOX-1 in more complex biological systems.

In future, we believe that the idea of SOS approach will be a useful tool for the discovery of new drug-like inhibitors, not only for 15-LOX-1 but for different protein targets as well. We believe that our improved 15-LOX-1 inhibitors and our new activity-based probes, could point out the way toward the development of therapeutic agents against diseases with an inflammatory component, such as asthma and COPD as well as neurological disorders like Alzheimer's and Parkinson's disease. For example, Alzheimer's disease effect millions of people while no treatments stop or reverse the disease progression though some may temporarily improve symptoms. Could 15-LOX-1 be a new hope for Alzheimer's disease? We think, yes. So, we created and now offer the tools to further study this hypothesis.