

University of Groningen

Modulation of lipoxygenase activity and chemistry-based detection of protein nitration in inflammation

Wisastra, Rosaline

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version

Publisher's PDF, also known as Version of record

Publication date:
2013

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Wisastra, R. (2013). *Modulation of lipoxygenase activity and chemistry-based detection of protein nitration in inflammation*. s.n.

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

**Summary,
General Discussion and
Future Perspectives**



6

SUMMARY

Modulation of Lipoxygenase activity by Anacardic acid Derivatives

Inflammation is a major health problem in the western world. This disease is influenced by numerous signaling molecules and signal transduction pathways. The NF- κ B signaling pathway is one of the main pathways that is involved in the regulation of gene transcription in inflammation and cancer. Furthermore, the activation and suppression of the NF- κ B signaling pathway are related to the activity of inflammatory enzymes such as for example lipoxygenases.

Lipoxygenases (LOXs) are non-heme iron containing enzymes that catalyze the oxidation of cis-unsaturated fatty acids. LOXs are divided into several sub-families such as 5-LOX, 8-LOX, 12-LOX and 15-LOX based on their selectivity to oxidize cis-unsaturated fatty acids. These oxidized fatty acids are mediators that are crucial for the initiation and also the termination of inflammatory processes. In addition, increasing evidence shows that the development of different types of cancer is associated with the activity of LOXs and the presence of their metabolites. Due to their relevance in diseases, the development of small molecule modulators of LOX enzymes is important. These molecules can be employed as tools to investigate these enzymes in their physiological and pathological context. Moreover, small molecule modulators of enzyme activity can be employed as starting points to develop new therapeutic approaches.

In **chapter 2** we describe the synthesis of a set of anacardic derivatives in which the substituents in the 4- and 6- position of the salicylate core were varied. The compound collection was screened *in vitro* for inhibition of soybean 15-lipoxygenase-1 (SLO-1) and potato 5-lipoxygenase (potato 5-LOX) to determine structure-activity relationships. We identified compound **2.23**, with a hydroxyl group and a 4-heptylphenethyl substituent, respectively in the 4- and 6- position of the salicylate core, as the most potent inhibitor for SLO-1. This compound gives a fivefold improvement of the inhibitory potency compared to anacardic acid. Kinetic studies of this compound on SLO-1 were performed in order to resolve its binding mechanism. Compound **2.23** shows a mixed type of inhibition, which indicates that this inhibitor binds to the free enzyme as well as to the substrate-bound enzyme. Most likely this inhibitor binds to an allosteric binding pocket, which regulates the catalysis in the active site of the enzyme.

Interestingly, another derivative **2.21** shows a strong activation of potato 5-LOX with about three fold activation at concentrations higher than 50 μ M. A kinetic study on potato 5-LOX indicates that this compound is a non-essential activator of the enzymatic reaction, which can occur in the presence or in the absence of activator. A comparable kinetic model as used for the mixed type inhibition of SLO-1 can be employed to derive the binding constants. Activator **2.21** shows a relatively high affinity for the substrate bound enzyme with the αK_A of 24 μ M and enhances the enzymatic conversion of the substrate. The kinetic analysis for SLO-1 and potato 5-LOX indicates the presence

of a regulatory site in the lipoxygenases that is targeted by anacardic acid and its derivatives. This provides a novel concept for modulation of lipoxygenase activity.

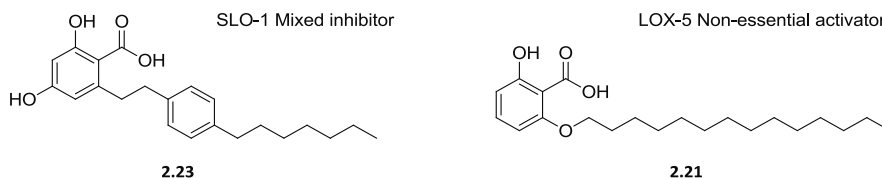


Figure 1 – Inhibitor and activator of SLO-1 and potato LOX-5 as described in chapter 2.

The anacardic acid structure consists of a hydrophilic salicylate core and a hydrophobic aliphatic chain and is thus expected to have surfactant characteristics, which can influence the biological potency by micelle formation. Hence, we investigated the critical micelle concentration (CMC) for anacardic acid, compound **2.21**, and compound **2.23**. The CMC values proved to be higher than 100 μM , which indicates that micelle formation does not play a role at concentrations that were used to investigate enzyme inhibition or activation.

We continued our investigations on anacardic acid and its derivatives as lipoxygenase modulator on human enzymes. In **chapter 3**, we screened a collection of compounds based on anacardic acid for inhibition of human 5-lipoxygenase (h-5-LOX) and compared it to cyclooxygenase-2 (COX-2) inhibition, which is another oxidative enzyme that plays an important role in inflammation and cancer. Anacardic acid derivatives show selective modulatory activity against h-5-LOX compared to COX-2. Compound **2.23** and compound **2.21** which in this chapter were labeled as compound **3.19** and compound **3.21** show no activation and/or inhibition on h-5-LOX and COX-2. These results demonstrate a different behavior between human lipoxygenase and plant lipoxygenase, which is most likely due to a lack of similarities in their primary structure. However, activation of h-5-LOX was observed from another anacardic acid derivative, compound **3.23a**. This compound gives 150% activation of h-5-LOX at a concentration of 12.5 μM . Kinetic studies for this compound on h-5-LOX reveal that compound **3.23a** is a non-competitive activator against the h-5-LOX co-activator ATP and a mixed non-essential activator against the h-5-LOX substrate linoleic acid. The mixed non-essential type of activation indicates the presence of an allosteric binding site that regulates the enzyme activity. The non-competitive behavior with respect to ATP demonstrates that this regulatory site is distinct from the ATP binding site. Remarkably, the h-5-LOX activator binding constant for **3.23a** is in the nano-molar range (381 nM).

Interestingly, a comparable compound (**3.23d**) shows inhibition of 5-h-LOX activity. The enzyme kinetics demonstrates mixed-type inhibition, which is comparable to the mechanism of action of activator **3.23a**. This supports the idea that these compounds bind to a regulatory site in h-5-LOX, which influences the catalytic processes in the active site. This study indicates the presence of an allosteric regulatory site that either inhibits or activates enzymatic catalysis based on the structure of the compound that binds to it.

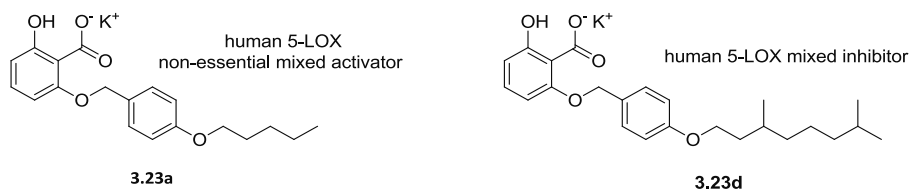


Figure 2 – Inhibitor and activator of human 5-lipoxygenase as described in chapter 3.

Human 5-lipoxygenase has important roles both in the initiation and termination of inflammatory responses; therefore the modulation of this enzyme is an interesting target for the development of novel therapeutic approaches. Due to the broad regulatory scope of h-5-LOX metabolites in cancer and inflammation both inhibitors and activators of this enzyme could provide interesting biological effects *in vivo*, which might ultimately be employed for therapeutic purposes.

Several inhibitors for lipoxygenases have been identified and some of them are under investigation in clinical trial as drugs for treatment of inflammatory diseases. Several inhibitors for the 5-lipoxygenase sub-family have been clinically used for asthma and arthritis. However, their use is still limited by a lack of efficacy, selectivity and cell permeability. Considering LOXs important role in inflammation and cancer, the development of novel classes of small molecule inhibitors of LOXs with higher selectivity profiles and alternative sub-types selectivities remains necessary.

The studies described in this thesis are focused on the development of chemical tools that can be used in order to apprehend the regulation of inflammatory processes in association with the NF- κ B signaling pathway. In the first part of this thesis, we develop derivatives of the natural product anacardic acid as novel modulators of lipoxygenase activity. In our first study (**chapter 2**), we identify an anacardic acid derivative that selectively inhibits soybean lipoxygenase-1 (SLO-1) and another derivative that activates potato 5-lipoxygenase (potato 5-LOX). Our subsequent study (**chapter 3**) on human 5-lipoxygenase (h-5-LOX) shows that plant enzymes and human enzymes are modulated by different derivatives. This study led to the identification of a novel inhibitor for 5-lipoxygenase, which is selective in comparison with cyclooxygenase-2 (COX-2). In addition, we identified a strong activator for h-5-LOX. Investigations of the effects on the h-5-LOX inhibitors and activators on signaling via the NF- κ B pathway are in progress.

We anticipate that both inhibitors and activators of h-5-LOX may find applications in distinct therapeutic areas. Inhibition of h-5-LOX has been associated with the suppression of immune responses. In contrast, activation of h-5-LOX would be associated with the activation of immune responses. We anticipate, however, that activation of h-5-LOX might also have beneficial effects in inflammation since the production of lipoxins by lipoxygenases contributes to the termination of immune responses. This argues for a more comprehensive understanding of the signaling properties of lipoxygenase metabolites in their physiological context.

Isothiazolones as novel ABPP probes for thiol-dependent enzymes

It has been shown that histone acetylation plays a crucial role in the activation of inflammatory gene transcription through the NF- κ B pathway. In previous studies it has been shown that isothiazolones are small molecule inhibitors of the HAT PCAF and also induce apoptosis in different cancer cell lines. In **chapter 4** of this thesis, we explore the reactivity of isothiazolones towards thiolates and the selectivity of this compound class for non-related thiol-containing enzymes. In this case we investigated the selectivity between the HAT PCAF and the cysteine-protease cathepsin B. These studies provide a starting point to develop this compound class further for the application as probes for activity based protein profiling (ABPP) for thiol-containing enzymes.

In this study we observed that N-aliphatic substituted 5-chloroisothiazolone **4.11** is the most potent inhibitor for cathepsin B. Also of interest is that the disulfide **4.7** had a remarkably high potency for cathepsin B, whereas it did not inhibit the HAT PCAF. In addition, we observed that a selective HAT PCAF inhibition compared to cathepsin B can be obtained with N-aryl substituent 3-Cl-4-F-phenyl **4.9**. In a conclusion, isothiazolones are inhibitors for cysteinyl cathepsin B and HAT PCAF and their potency and selectivity depend on their substitution pattern.

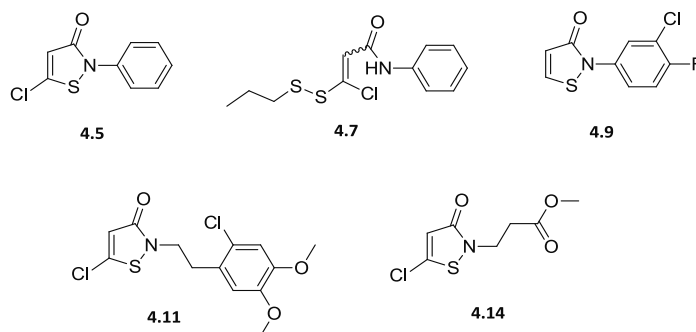


Figure 3 – Inhibitor of cysteine protease cathepsin B as described in chapter 4.

The reactivity of isothiazolones and 5-chloroisothiazolones towards thiolate originates from the electronic character of the isothiazolones heterocycle. Crystal structures of isothiazolone and 5-chloroisothiazolone show an aromatic character of the heterocycle, which provides stability to the compounds; in contrast the weak sulfur-nitrogen bond allows the reaction with the thiolate to form a strong disulfide bond.

We analyzed cathepsin B inhibitory mechanism of 5-chloroisothiazolones **4.5** and **4.14** through the enzyme kinetics. These studies show non-competitive inhibition of cathepsin B by 5-chloroisothiazolones and a strong substrate inhibition occurs at a higher concentration. Inhibition of cathepsin B by 5-chloroisothiazolone **4.5** proved to be time dependent and show a decreasing IC_{50} values overtime, which indicate a fast binding event followed by a slow second binding event. These data indicate a covalent binding of 5-chloroisothiazolones to cathepsin B. Furthermore, mass spectroscopy analysis shows that 5-chloroisothiazolone **4.5** binds once or twice to the enzyme, which

indicates that 5-chloroisothiazolone **4.5** binds covalently to the thiolate in the enzyme active site and/or to another surface exposed cysteine of cathepsin B. This study sets the stage to develop the isothiazolones further for applications as probes for activity-based protein profiling of HATs and cysteine proteases.

Detection of nitrotyrosine as inflammatory biomarker

Activation of inflammatory pathways leads to oxidative stress and subsequent damage of proteins by reactive oxygen species (ROS). An important oxidative protein modification is nitration of tyrosine residues, which has been identified as a biomarker for several inflammatory diseases. In the last part of this thesis (**chapter 5**), we describe the development of a novel method to detect nitrotyrosine containing proteins in biological samples. This novel method involves the conversion of nitrotyrosine into a fluorophore under mild conditions. This novel method is easy to perform, highly selective for nitrotyrosine and relies on cheap reagents. It proved to be very efficient in histochemical staining of inflamed tissue and can also be employed as alternative detection for immunoblotting on PVDF membranes. As expected, the sensitivity of this novel fluorescence based detection method is lower than for enzyme linked detection methods, which include enzyme-catalyzed signal amplification.

The method implies two reaction steps; the first step is a conversion of the 2-nitrophenol functionality into a 2-aminophenol functionality using sodium dithionite, and the second step is a reaction of the 2-aminophenol functionality with Al^{3+} and salicylaldehyde to form a fluorophore.

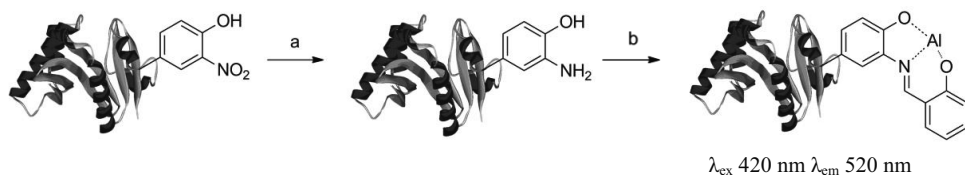


Figure 4 – Fluorescence detection of nitrotyrosine in a tissue slice; reduction of a protein bound 2-nitrophenol functionality to a 2-aminophenol functionality: a) aqueous $\text{Na}_2\text{S}_2\text{O}_4$; subsequent conversion into a fluorophore: b) aqueous AlCl_3 and salicylaldehyde.

The fluorescence of the 2-aminophenol - Al^{3+} - salicylaldehyde complex in demiwater is 8 times higher than in methanol. Detection in water is the most favorable condition for detection of protein nitration in biological samples. Furthermore, we investigated the application of this novel method for histochemical staining in comparison with immunohistochemical staining in different tissue samples. Remarkably, this novel method shows a clear staining in liver tissue section from CCl_4 treated mice, which is a model for inflammation. The novel histochemical staining was compared to the classical immunohistochemical staining. Both staining methods showed high intensities in the same locations of tissue samples; however the classical histochemical staining shows a much higher background staining. This clearly demonstrates that our newly developed histochemical staining method is, in this respect, superior to the classical immunohistochemical staining. In addition, the formed fluorophore in tissue sample was stable for several days with proper storage conditions. These results indicate that

this novel histochemical staining method can provide a valuable alternative for detection of protein tyrosine nitration in tissue samples with high stability upon prolonged storage and lower background fluorescence signals.

We also evaluated the application of this novel method for fluorescence detection in biological matrices on western blot. 3-nitro-4-hydroxy phenyl acetic acid (NHPA) was coupled to bovine serum albumin (BSA) as a model for tyrosine nitrated proteins. This NHPA-conjugated BSA was used as a standard to optimize the detection method. Our novel staining method enabled the detection of NHPA-conjugated BSA on western blot. Control experiments with the classically used anti-nitrotyrosine antibody provided comparable results. The presence of blood plasma did not significantly alter the fluorescence signal, which demonstrates the applicability of this novel method to detect 2-nitrophenol in the complex biological matrices. The detection limit for this novel method is 500 nmol/L, which is about 20 times less sensitive compare to antibody detection method, which relies on enzymatic amplification. Due to the lower sensitivity no signal was detected from blood samples before and after immunoprecipitation enrichment using the novel fluorescence staining, whereas bands from nitrotyrosine enriched blood samples were observed from the antibody staining method. We conclude that the novel fluorescence method can be employed as an alternative staining method for 2-nitrophenol functionalities in the complex biological matrices.

Overall, this thesis provides a contribution to a better understanding of inflammatory responses by the development of novel chemistry-based tools for inhibition and detection of enzyme activity. Ultimately, these methods could be employed in diagnosis and therapy.

FUTURE PERSPECTIVES

Future studies should be focused on the improvement of inhibitors of lipoxygenases in terms of potency, selectivity, efficacy, and cell permeability. We identified anacardic acid derivatives as modulators of lipoxygenases with medium to high potency. Here, we revealed a regulatory mechanism of lipoxygenase activity that is influenced by salicylate-based molecules that bind to the allosteric site of these enzymes. This allosteric site that is targeted in h-5-LOX is different from the ATP binding site in The next challenge is to elucidate the location and the structure of this regulatory site in order to get a model for designing new modulators for this enzyme. The crystal structure of lipoxygenase in complex with the allosteric modulators may provide the necessary information on this allosteric site. Based on the crystal structure of the enzyme, the configuration of salicylate based modulators can be resolved and this information can be further used to design new compounds with improved inhibitory potency. In our studies, anacardic acid derivatives were shown to selectively inhibit h-5-LOX in comparison to COX-2 which provides a good basis for a new therapeutic approach. Furthermore, screening and kinetic studies against other LOXs and COXs enzymes will provide a more complete understanding of the selectivity of this set of compounds

The roles of lipoxygenase in inflammation and cancer are associated with the activation of NF- κ B pathway and the expression of pro-inflammatory genes. The identified modulators of LOX activity described in this thesis can be employed as tools to explore the connection between the lipoxygenase activities and activation of the NF- κ B pathway. Experiments on the potency of lipoxygenase activators and inhibitors to regulate the NF- κ B pathway are currently ongoing. Such studies can further confirm the importance of LOXs inhibitors as a therapeutic approach in inflammation and cancer.

We also briefly described the role of COXs in inflammation. Inhibition of COX-2 activity has frequently been employed for the development of anti-inflammatory drugs. Non-selective COXs inhibitors such as aspirin can provide moderate to severe side effects by inhibition of COX-1. Therefore the development of selective inhibitors became a major concern for this enzyme. In chapter 3 we describe the benzoxazole core scaffold as a potential starting point for development of a new class of inhibitors. Further studies on the selectivity of these compounds against COX-1 and the other LOXs are needed for further development of this compound class.

Compounds that covalently bind with thiolates have been employed frequently as starting points to develop ABPP probes for a cysteine containing enzymes such as HATs and cysteine proteases. We revealed the reaction mechanism of isothiazolones with thiolates, and our enzyme kinetic studies in combination with mass spectroscopy analysis showed that isothiazolones and 5-choloroisothiazolones bind covalently to the active site thiol and/or to another surface exposed cysteine of cysteine protease cathepsin B. This sets the stage for development of ABPP probes based on isothiazolones that can be targeted either to HATs, to cysteine proteases and potentially also other thiol-containing enzymes.