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Activity-Based Probes for 15-lipoxygenase-1

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Abstract

Human 15-lipoxygenase-1 (15-LOX-1) plays an important role in several inflammatory lung diseases such as asthma, COPD and chronic bronchitis as well as in various CNS diseases like Alzheimer's, Parkinson's and stroke. Activity-based probes of 15-LOX-1 are required to explore the role of this enzyme further and to enable drug discovery. In this study, 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. Mimicking the natural substrate of the enzyme, we designed activity-based probes that covalently bind to the active enzyme and include a terminal alkene as chemical reporter for bioorthogonal linkage of a detectable functionality via the oxidative Heck reaction. We believe that the activity-based labeling of 15-LOX-1 will enable the investigation and identification of this enzyme in complex biological samples, which opens completely new opportunities for drug discovery.

Keywords

15-LOX-1; activity-based probes; Kitz-Wilson plots; enzyme kinetics; irreversible inhibition

Activity-based protein profiling (ABPP) has become a powerful method for the analysis of enzyme function and the selectivity of enzyme inhibitors in complex disease models.[1] In ABPP, small molecule substrate analogues, known as activity-based probes, are used to covalently bind to the active site of enzymes depending on their activity. Currently, many research groups use ABPP to investigate various enzyme classes, like cysteine proteases[2–4], serine hydrolases[5,6], diacylglycerol lipase-a[7], glyceraldehyde 3-phosphate dehydrogenases[8], protein kinases[9], monoamine oxidases[10] and several others. However, probes are missing for Lipoxygenases (LOXs), which are important enzymes involved in diseases with an inflammatory component.

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The key role of LOXs in many disease processes originates from their metabolic activity on polyunsaturated fatty acids (PUFAs) such as arachidonic and linoleic acid. Through a radical mechanism, LOXs catalyze the regio- and stereospecific insertion of molecular oxygen (O_2) into PUFAs, resulting in the production of lipid signaling molecules.[11] Based on the position of O_2 insertion in arachidonic acid LOXs are classified as 5-, 8-. 12-, or 15-LOXs. Metabolites originating from LOXs activity such as leukotrienes and lipoxins exert versatile regulatory roles in the immune system.

In our study, human 15-lipoxygenase-1 (15-LOX-1) was selected as a starting point to develop activity-based probes for this class of enzymes. 15-LOX-1 is an important mammalian lipoxygenase that plays a role in the biosynthesis of 15-HPETE, 15-HETE, leukotrienes, lipoxins and eoxins. Several studies describe a role for 15-LOX-1 in allergic airway diseases[12–15], chronic airway inflammation[16], atherosclerosis[17], cancer[18,19] and more recently in various CNS diseases[20–29] like Alzheimer's and Parkinson's diseases as well as stroke. Therefore, this enzyme gained attention as a potential drug target and several classes of inhibitors have been described.[22,24–27,30–38]

Development of activity-based probes for target enzymes start from irreversible mechanism-based enzyme inhibitors. For soybean lipoxygenase, structural analogs of PUFAs have been reported in which the cis-alkenes are replaced by alkynes that proved to be irreversible inactivators of LOX enzymes.[39,40] Inactivation is expected to proceed through single electron oxidation of the bis-propargylic carbon resulting in an allene radical that is highly reactive and binds covalently to the enzyme's active site. We aimed to use this type of inhibitor as starting structure to develop activity based probes for LOX enzymes.

Mimicking the natural 15-LOX-1 substrate, linoleic acid, inhibitors were designed incorporating a bis-alkyne core structure and their binding properties were investigated. [39,40] After modeling studies, in contrast with the previous inhibitors we shift the position of the bis-alkyne moiety from 9,12 to the 5,8 position due to structural differences in the active sites between the two enzymes (Figure S9) but also aiming to yield less lipophilic compounds. Next, we developed ABPP probes that include both a bis-alkyne functionality for covalent linkage to the active enzyme and a terminal alkene as chemical reporter for bioorthogonal linkage of a detectable functionality (Figure 1). Application of a terminal alkene as chemical reporter and not the more commonly used terminal alkyne enables straightforward synthesis of the ABPP probe using methods shown in Figure 2 without the need for protection and deprotection of the reporter functionality. As demonstrated recently, terminal alkenes can be linked to biotinylated phenylboronic acid by application of the recently developed bioorthogonal oxidative Heck reaction,[41,42] which proceeds under mild conditions. Using these methods we demonstrate for the first time activity-based labeling of lipoxygenase activity, which paves the way for exploration of this novel area.

As a first step a small library of bis-alkyne inhibitors, with aliphatic chains of various lengths, was synthesized in order to investigate the effect of lipophilic interactions on the binding affinities and inactivation kinetics of 15-LOX-1 (Figure 3A). The bis-alkyne inhibitors were synthesized by K_2CO_3 mediated CuI-catalyzed cross-coupling of methyl 5-hexynoate with various propargylhalides, in presence of NaI. The applied propargylhalides

were either commercially available or synthesized starting from propargyl alcohol in four steps in good yields (Figure 2). Firstly, propargyl alcohol was protected with tert-butyl diphenylsilyl chloride (TBDPS-Cl) to give TBDPS protected compound **1**. The protected propargyl alcohol was then coupled with different aliphatic bromides in presence of *n*-BuLi and HMPA at -78 °C to afford, after deprotection with TBAF in THF, the corresponding propargyl alcohols **2**. Finally, propargylhalides **3** were isolated after bromination of the alcohols **2** with CBr_4 and PPh_3 in benzene at 0 °C and subsequently applied in the cross-coupling reaction.

The newly synthesized bis-alkynes were screened for inhibition of 15-LOX-1 as described before.[38,43,44] IC_{50} determination of all the compounds showed potencies in the low micromolar range (Figure 3A). Bis-alkyne inhibitors with longer aliphatic chains seem to be the more potent, probably due to lipophilic interactions. Notable is that the IC_{50} values for all the compounds proved to be time dependent with a slight difference between 10 and 20 min preincubation time, which indicates irreversible inhibition. Further analysis using Lineweaver-Burk plots showed non-competitive inhibition for inhibitor **N144**, which also supports a model in which the inhibitors bind irreversibly (Figure 3D).

Further analysis of the binding kinetics was done using Kitz-Wilson analysis to derive the inactivation parameters K_i and k_i (Figure 3C).[45–47] Four concentrations of the respective inhibitor and four pre-incubation times with the enzyme were chosen to measure dose and time dependent inhibition of 15-LOX-1 activity. The inactivation parameters for all the bis-alkyne inhibitors were calculated (Figure 3A). The K_i values vary between 40 μM to 15 μM, with the shorter inhibitors (**ST018**, **ST022** and **ST025**) being less potent than those with five to seven member carbon chains (**N86**, **ST024**, **N121**, **N331**, **N332**, **N333** and **N144**). The inactivation rate (k_i) values range from 0.05 min^{-1} to 0.35 min^{-1}, showing a reaction half time ($t_{1/2}$) from 2 to 12 min. In conclusion, the kinetic analysis supports a model in which bis-alkyne inhibitors bind irreversible to 15-LOX-1. Molecular modeling studies on this type of inhibitors provided a model in which the bis-alkyne moiety of the compounds appear close to the iron in the active site (Table S5 and Figure S6,S7). Considering the enzyme kinetics and molecular modeling studies as well as the polarity of the compounds, chain length of inhibitor **N86** was chosen to design probes for activity-based labeling of lipoxygenases.

As a next step **N86** was modified with a terminal alkene as bioorthogonal tag to result in compounds **N144** and **N121** (Figure 3A). These molecule were applied in ABPP labeling experiments on the recombinant purified enzyme 15-LOX-1. Because of the observed fast inactivation $t_{1/2}$, the labeling experiments were done in a relatively short time (2 min). The ABPP labeled enzyme was detected using covalent attachment of biotinyl phenyl boronic acid to the terminal alkene chemical reporter using the oxidative Heck reaction[42,48] and subsequent visualization by on blot luminescence imaging using HRP-conjugated Streptavidin. In the oxidative Heck reaction it proved to be important to use 10% DMF as co-solvent in the reaction mixture to avoid non-specific binding of biotinyl phenyl boronic acid to the protein. Application of this method to recombinant 15-LOX-1 provided clear labeling of the enzyme with probe **N144** in comparison to the control experiment in which this probe was excluded (Figure 4A), whereas a control experiment with a 15-LOX antibody

demonstrated equal amounts of the enzyme. Probes **N144** and **N121** were compared and **N144** provided better labeling efficiency in comparison to probe **N121** (Figure S13). This demonstrates that the enzyme 15-LOX-1 can be covalently labelled and that this two-step labeling approach enables visualization of this enzyme on Western blot.

Having established 15-LOX-1 labeling, we moved on to investigate its dependence on LOX enzyme activity. Experiments with heat inactivated 15-LOX-1 in comparison to active 15-LOX-1 demonstrated a clear difference in labeling as compared to the control in which the probe **N144** was excluded from the experiment (Figure 4B). Additionally, 15-LOX-1 was subjected to small molecule inhibition by the known reversible selective 15-LOX-1 inhibitor, **PD-146176**.[30] As a control we included the inhibitor **Zileuton (Zyflo)**, which is a known reversible and selective 5-LOX inhibitor. The 15-LOX-1 enzyme was incubated with either **PD-146176** or **Zileuton** for 10 min, followed by 2 min incubation with the probe and subsequent biotinylation. The enzyme labeling was assessed on Western blot using equal amounts of the enzyme. Only in the case of **PD-146176** labeling was clearly inhibited compared to the positive control (without inhibitor), whereas **Zileuton** did not affect the labeling of 15-LOX-1 (Figure 4C). Taken together, these experiments confirm that the bis-alkyne **N144** can be used for activity-based labeling of recombinant purified 15-LOX-1.

After having established 15-LOX-1 labeling and its activity dependence on the purified enzyme, we continued labeling experiment with more complex biological samples. Firstly, lysates of HeLa and IL-4 stimulated HBE cells proved to contain 15-LOX-1 (as demonstrated by Western blotting). HBE cells have been reported to have higher expression of the enzyme after IL-4 stimulation.[49] Lysates from these samples were labeled with **N144** for 2 min followed by biotinylation via the oxidative Heck reaction. The activity-based labeling showed pronounced and distinct bands that were not visible in the negative control in which the probe was excluded (Figure 5A,B). Notably, the labeling of 15-LOX-1 in HBEs has a different pattern as compared to the labeling in HeLa cells. Nevertheless, both lysates show labeling at 70 kDa, which is the expected molecular weight for 15-LOX-1. In addition, a clear concentration dependence was observed for the labeling in HeLa cell lysate using increasing concentration of the probe **N144** (Figure 5C). Subsequently, the HeLa lysate was evaluated for the presence of 15-LOX-1 in combination with the activity-based labeling. Western blotting using a 15-LOX-1 antibody demonstrated that the antibody recognized four bands (Figure 5D). One of the bands appears in the expected weight for 15-LOX-1 (70 kDa) and two bands were lower. These lower bands either originate from degradation of the 15-LOX-1 enzyme or represent other proteins (Table S6,7). Subsequently, the membrane was stripped and the activity-based labeling was detected using HRP-conjugated streptavidin. This again displayed the bands for the activity-based labeling. After the 15-LOX-1 antibody detection and stripping in Figure 5D the band proved to become less clear compared to the immediate detection in Figure 5A. The apparent 15-LOX-1 band at 70 kDa as well as the other two proteins nicely align with bands visible in the activity-based labeling experiment. One of these proteins also appears in the same range (above 40kDa) in the HBE lysates (Figure 5B). In addition, labeling experiment after incubation of the probe **N144** with intact HeLa cells was also performed successfully (Figure S25). These experiments demonstrate activity-based labeling on purified or endogenous 15-LOX-1 from HeLa or HBE cell lysates.

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In order to investigate the 15-LOX-1 activity dependence of the labeling, we labeled active and inactive recombinant 15-LOX-1 in presence of heat denaturated cell lysate. We observed that the heat inactivated lysates were not labeled whereas the heat inactivated lysates supplemented with active 15-LOX-1 show labeling of just 15-LOX-1 (Figure S21). Next, we applied pharmacological 15-LOX-1 inhibition with inhibitor **PD-146176** in the labeling experiment with HeLa cell lysates. We observed a decrease of the labeling in the bands that were also characterized by the 15-LOX-1 antibody (Figure 5E). This indicates that the labeling of these bands is activity-dependent.

Finally, the activity-based probe has been applied in tissue lysates from different mice organs. Applying probe **N144** and following our two-step labeling, we were pleased to observe a clear labeling of 15-LOX-1 in different tissue lysates (Figure 5F). The different band intensities indicate different enzyme expression and activity levels in the different organs, which is an observation that is of particular interest for drug discovery projects aimed at targeting this enzyme. In addition, we noted that when fresh tissue samples were used, the labeling pattern became more clear, indicating that the lipoxygenases seem to be unstable under the storage conditions. Furthermore, we evaluated inhibitors **PD-146176** and **Zileuton** in heart lysate in more detail. The results showed again reduced labeling of the three characteristic bands only upon application of the 15-LOX-1 inhibitor **PD-146176** (Figure 5G), which indicates that the observed labeling originates from 15-LOX-1 activity in this tissue sample.

In conclusion, 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. We anticipate that further development of this type of molecules will enable the investigation and identification of lipoxygenase enzymes in complex biological samples, which opens completely new opportunities for drug discovery for this enzyme class.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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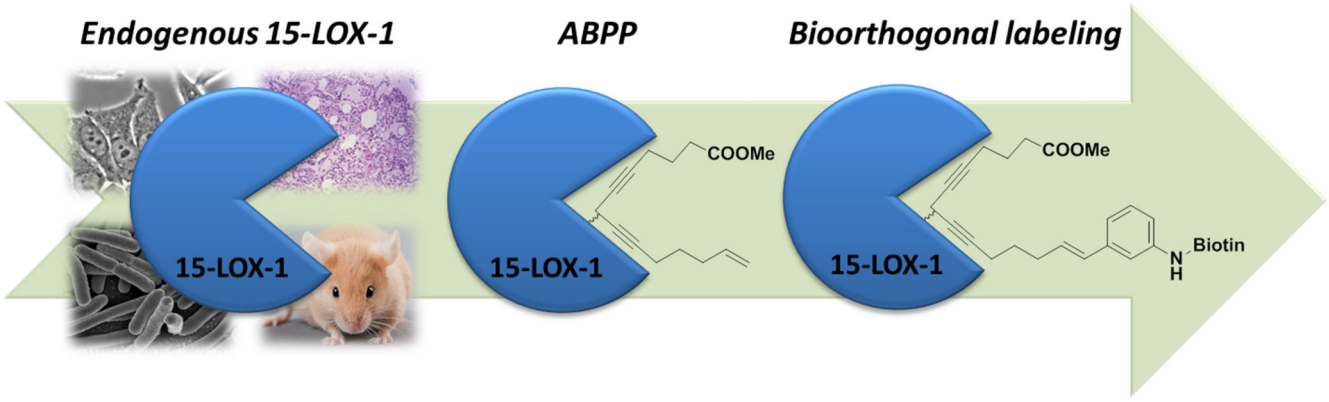

Figure 1.

Two step identification of 15-LOX-1 using ABPP. The labeling of 15-LOX-1 was performed after two minute incubation with the activity-based probe followed by biotinylation via oxidative Heck reaction.

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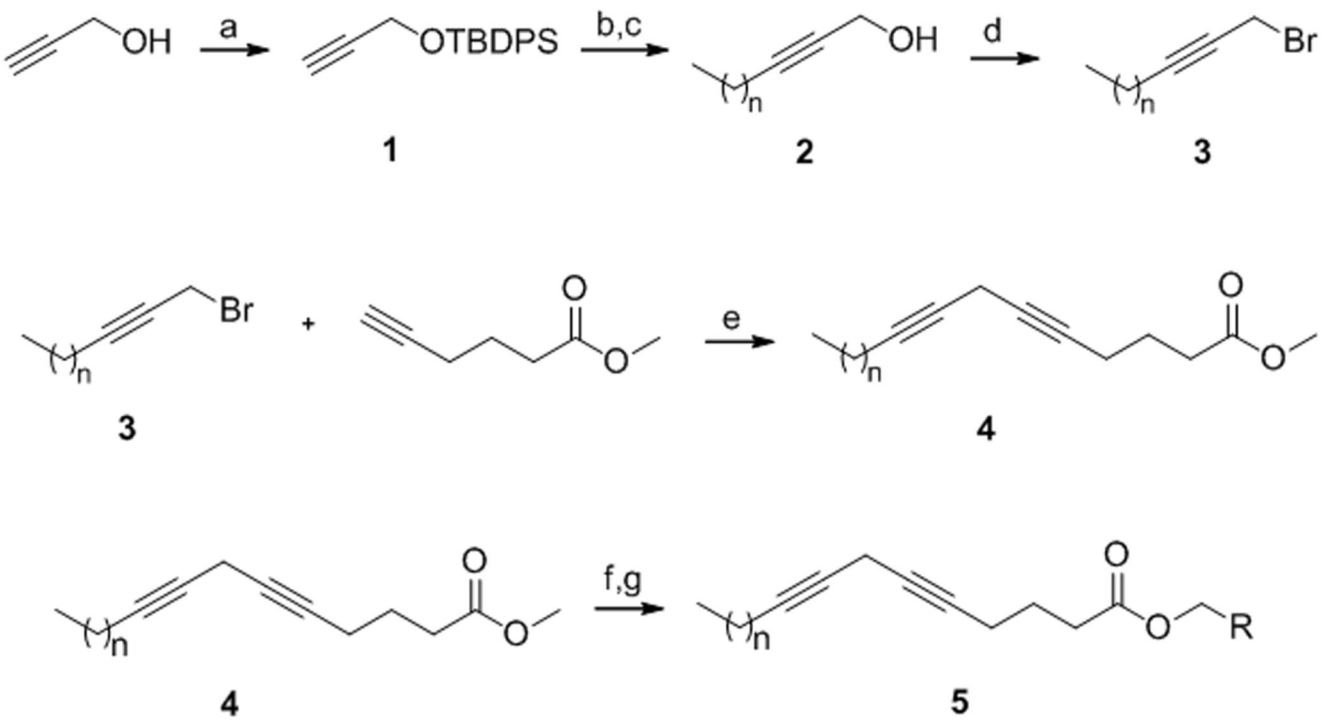

Figure 2.

Synthesis of irreversible inhibitors and probes. a) TBDPSCl, imidazole, DMF, RT; b) Bromoalkanes or -alkenes, n-BuLi, HMPA, -78°C; c) TBAF, THF, 0°C; d) CBr_4, PPh_3, C_6H_6, 0°C; e) CuI, NaI, K_2CO_3, DMF, RT; f) LiOH, EtOH/H_2O (3:1), RT; g) Bromoalkanes or –alkenes, K_2CO_3, DMF.

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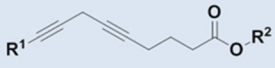
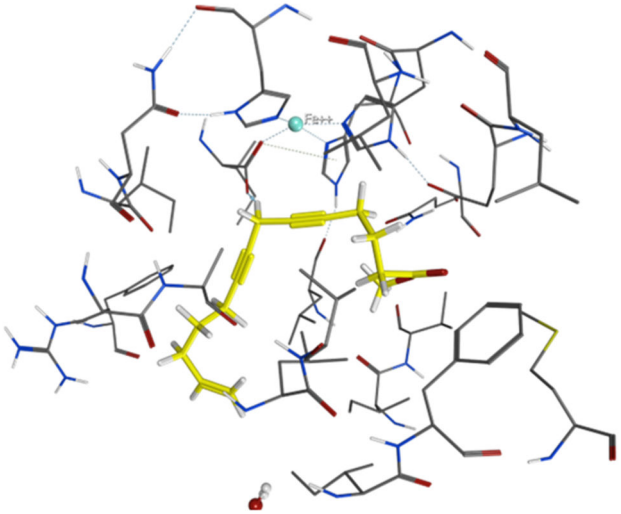
A

Structure	Compound	R^1	R^2	IC_{50} (μM)	ki (min^{-1})	Ki (μM)
	ST022	Me	Me	21.2 ± 2.7	0.109 ± 0.048	28.9 ± 6.5
	ST018	Et	Me	14.1 ± 2.6	0.350 ± 0.165	41.2 ± 7.8
	N86		Me	5.15 ± 0.62	0.130 ± 0.021	23.8 ± 5.8
	ST024		Me	5.86 ± 0.31	0.057 ± 0.009	22.1 ± 2.1
	N114		Me	7.04 ± 4.86	0.097 ± 0.036	32.8 ± 5.1
	ST025		Me	8.69 ± 1.23	0.156 ± 0.038	30.8 ± 2.7
	N144		Me	4.03 ± 0.45	0.156 ± 0.066	23.6 ± 3.2
	N121			2.17 ± 0.16	0.125 ± 0.042	20.7 ± 3.8
	N331			3.98 ± 0.54	0.153 ± 0.079	13.7 ± 7.1
	N332			5.64 ± 1.07	0.159 ± 0.085	15.6 ± 8.3
	N333			8.16 ± 1.08	0.157 ± 0.005	16.2 ± 0.5

B



C

$$E + I \underset{}{\overset{K_i}{\rightleftharpoons}} E \cdot I \xrightarrow{k_i} E\text{-}I$$

D

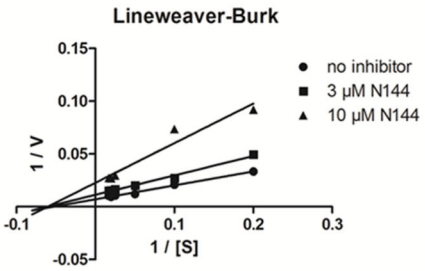


Figure 3.

A) Table with the synthesized compounds, their IC_{50} values (10 min) and inactivation parameters. All the values are reported with the standard deviation. B) Highest scoring docking pose of probe **N144** in the active site of 15-LOX. C) Irreversible inhibition equation. D) Lineweaver-Burk plot of probe **N144**.

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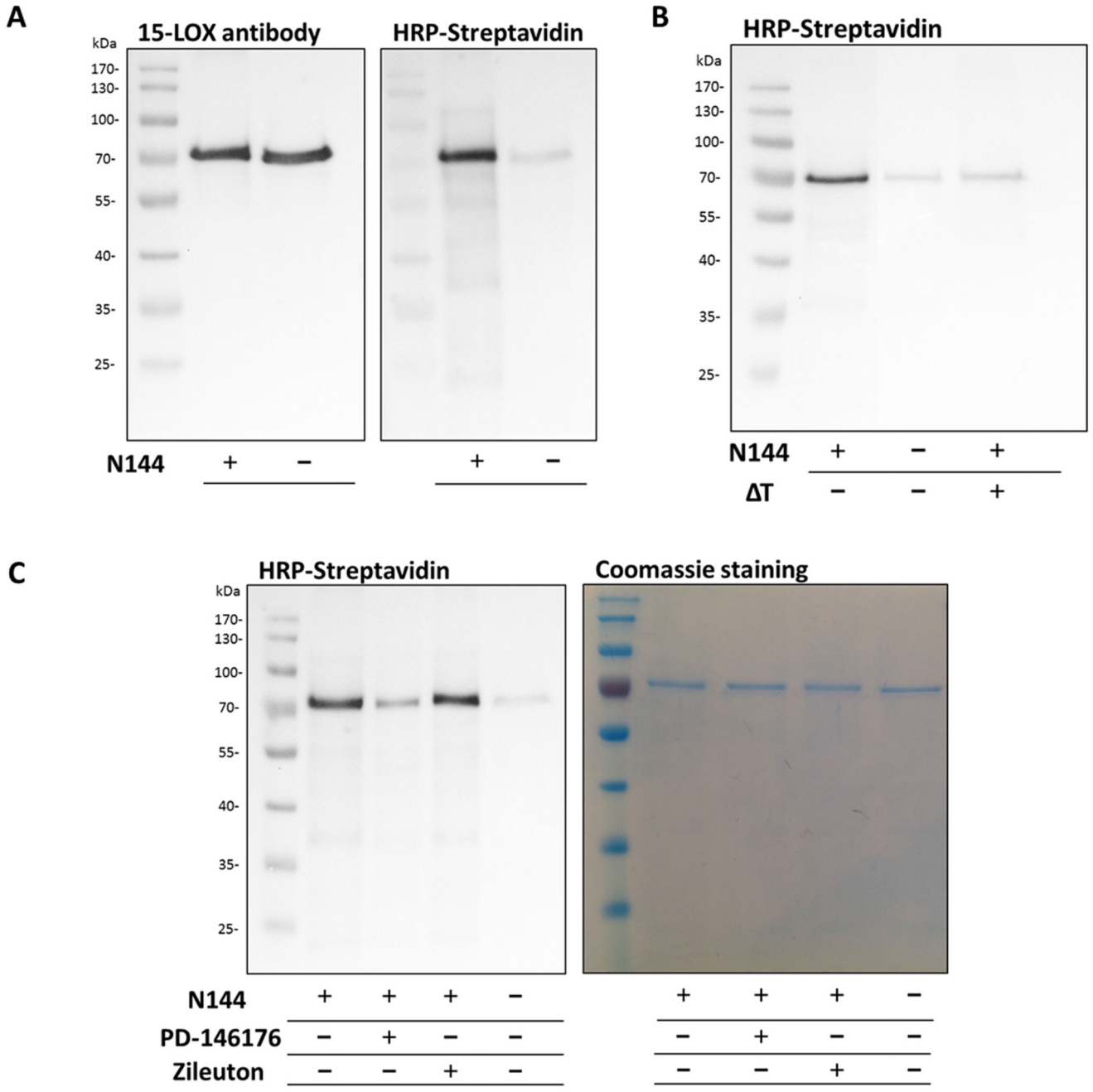

Figure 4.

On blot detection and Coomassie Blue staining of pure 15-LOX-1. A) Positive (with probe) and negative control (without probe) experiments using 15-LOX antibody or streptavidin as secondary antibody. B) Labeling of the enzyme after heat denaturation (right) and without heat denaturation (left). C) Labeling after pre-incubation of the enzyme with **PD-146176** and **Zileuton**.

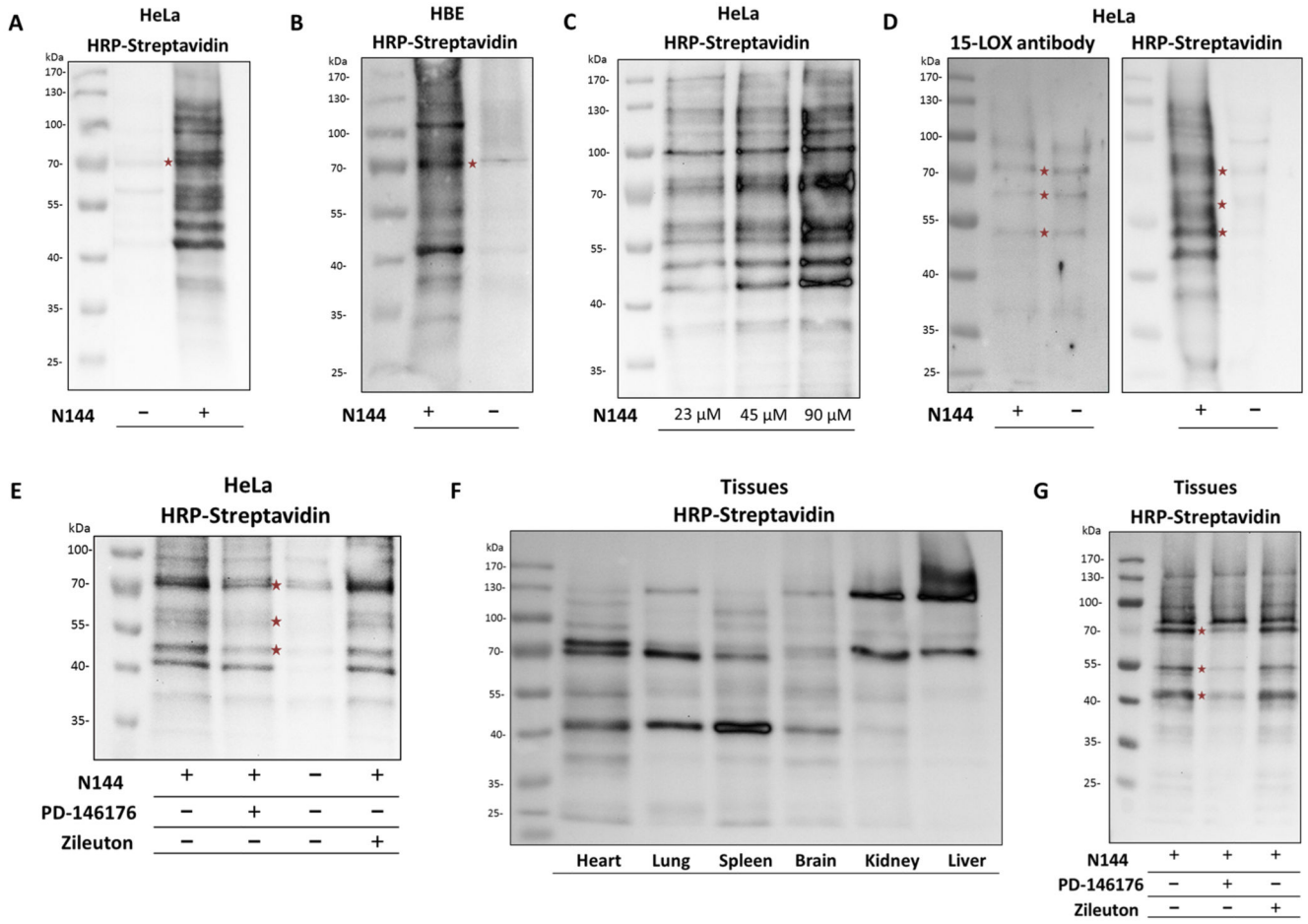

Figure 5.

On blot detection of endogenous 15-LOX-1 A) Positive (with probe) (right) and negative control (without probe) (left) using HRP-Conjugated Streptavidin in HeLa cells lysate. B) Positive (with probe) (left) and negative control (without probe) (right) using HRP-Conjugated Streptavidin in HBE cells lysate after IL-4 stimulation. C) Concentration dependent labeling by activity-based probe. D) Positive (with probe) and negative control (without probe) experiments using 15-LOX antibody and after stripping HRP-Conjugated Streptavidin as secondary antibody. E) Labeling after pre-incubation of the endogenous enzyme with inhibitors **PD-146176** (90 μM) and **Zileuton** (90 μM). F) Labeling in different mouse tissue lysates. G) Labeling of mouse heart lysate after pre-incubation of the endogenous enzyme with inhibitors **PD-146176** (90 μM) and **Zileuton** (90 μM).

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