Journal of Medicinal Chemistry

Article

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J. Med. Chem., Just Accepted Manuscript • DOI: 10.1021/acs.jmedchem.9b00212 • Publication Date (Web): 09 Apr 2019 Downloaded from http://pubs.acs.org on April 10, 2019

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is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

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A novel 15-Lipoxygenase-1 Inhibitor Protects Macrophages from Lipopolysaccharides-Induced Cytotoxicity

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Abstract

Various mechanisms for regulated cell death include the formation of oxidative mediators such as lipid peroxides and nitric oxide (NO). In this respect, 15-lipoxygenase-1 (15-LOX-1) is a key enzyme that catalyzes the formation of lipid peroxides. The actions of these peroxides are interconnected with nuclear factor- κ B (NF- κ B) signaling and NO production. Inhibition of 15-LOX-1 holds promise to interfere with regulated cell death in inflammatory conditions. In this study, a novel potent 15-LOX-1 inhibitor, **9c (i472)**, was developed and structure–activity relationships were explored. *In vitro*, this inhibitors protected cells from LPS-induced cell death, inhibiting NO formation and lipid peroxidation. Thus, we provide a novel 15-LOX-1 inhibitor that inhibits cellular NO production and lipid peroxidation, which set the stage for further exploration of these mechanisms.

1. Introduction

Over recent years, an increasing number of mechanisms for regulated cell death have been identified and versatile roles in numerous diseases were proposed.¹ Cell death *via* a mechanism other than apoptosis leads to plasma membrane rupture and release of the cellular content, thus providing damage-associated molecular patterns (DAMPs) that can induce an auto-amplification loop of regulated cell death and inflammation. Such amplification loops are expected to play key roles in diseases such as acute lung injury and acute respiratory distress syndrome.² Understanding the underlying mechanisms to develop of small molecule inhibitors to interfere with cell death holds promise for therapeutic control of these disorders.

The discovery of multiple types of cell death provides new challenges to identify the molecular mechanisms involved. One mechanism of non-apoptotic cell death is pyroptosis in which macrophages die by excessive stimulation of Toll-like receptors (TLRs) and activation of the NF- κ B pathway by, for example, lipopolysaccharides (LPS).^{2–6} Normally, pyroptosis is a mechanism to protect multicellular organisms from invading pathogens, such as microbial infections. However, under pathogenic conditions pyroptosis can be involved in the onset of chronic inflammation. Another mechanism for non-apoptotic cell death is ferroptosis, which is a process in which excessive levels of lipid peroxides cause cell death. It is anticipated that lipoxygenases (LOXs) play key roles in ferroptosis by catalyzing lipid peroxidation.^{2,7} The identification of pyroptosis, ferroptosis and other mechanisms for regulated cell death raises the question how these mechanisms can be exploited for drug discovery.

Although distinct mechanisms for regulated cell death were described, the mechanisms involved are often closely related and cross talk exists. In this study, we aim to address the cross-

talk between macrophages cell death upon LPS stimulation and the enzymatic activity of 15lipoxygenase-1 (15-LOX-1) as a regulator of cellular lipid peroxidation (Figure 1).⁸ Activation of the NF-κB pathway results in transcription of downstream genes, such as inducible nitric oxide synthase (iNOS), that plays a critical role in inflammatory responses.⁹ iNOS catalyzes the formation of NO radicals that play key roles in many physiological processes.¹⁰ On the other hand, excessive NO production can lead to the formation of reactive nitrogen species (RNOS), which induces cell death and tissue damage.¹¹

Reactive oxygen species (ROS) such as lipid peroxides have been shown to augment LPSmediated NF-kB activation and thus increase expression of NF-kB target genes,^{8,12} which represents a mechanism of crosstalk between lipid peroxidation and NF-kB activation. 15-LOX-1 is a non-heme iron-containing enzyme producing lipid peroxides from poly-unsaturated fatty acids (PUFAs), such as arachidonic acid (AA) and linoleic acid (LA).^{13–15} 15-LOX-1 oxidizes either AA, to form the corresponding 15-hydroxyeicosatetraenoic acid (15-HpETE), or LA, to form the corresponding 13-hydroperoxyoctadecadienoic acid (13-HpODE).^{16,17} Apart from these hydroperoxy fatty acids, lipoxins are also derived from the 15-LOXs pathway and play a role as anti-inflammatory mediators.¹⁸ On the other hand, the 15-LOXs metabolites, eoxins are proposed to be a family of pro-inflammatory eicosanoids.¹⁹ Altogether, lipid peroxides and can be converted further in distinct lipid signaling molecules that have key regulatory roles in immune responses.^{20–22} and numerous diseases.²³ Importantly, if the production of lipid peroxides is not balanced by the cellular anti-oxidant system, this can result in ferroptotic cell death and in enhanced activation of the NF-kB pathway, thus providing synergistic crosstalk between two mechanisms of regulated cell death.²⁴ Thus 15-LOX-1 is a key enzyme in oxidative stress and regulated cell death in numerous diseases.^{13,25,26}



Figure 1. Several mechanisms of lipopolysaccharide (LPS) signaling in macrophages are connected to cell death. LPSmediated activation of NF-κB pathway results in the overexpression of inducible nitric oxide synthase (iNOS). This leads to the production of nitric oxide (NO) and reactive nitrogen species (RNOS), which is involved in cell death. In the 15-LOX-1 pathway, 13-hydroperoxyoctadecadienoic acid (13-HpODE), the metabolite of 15-LOX-1 activity, can also induce cell death. Both mechanisms act in concert and cross talk exist.

For 15-LOX-1, roles have been described in diseases such as asthma,¹⁴ stroke,¹⁵ atherogenesis,² diabetes,^{16,17} cancer,^{20,21} Alzheimer's disease,^{22,23} and Parkinson's disease.²⁵ This triggered the interest in the development of 15-LOX-1 inhibitors for drug discovery. In an early phase, indole-based inhibitors, **PD-146176** was identified as r-12/15-LOX inhibitor with an IC₅₀ value of 3.81 μ M (Figure 2).²⁷ This stimulated efforts to develop inhibitors with an indolyl core (Figure 2). More researchers reported the discovery of indole-based or indole-like 15-LOX-1 inhibitors, **371** and Haydi-**4b** (with IC₅₀ of 0.006 and 3.84 μ M, respectively).^{28,29} In our group, we previously discovered 15-LOX-1 inhibitor Eleftheriadis-14d, which also contains an indole core and demonstrates good potency (IC₅₀ = 90 nM).³⁰ Furthermore, 1,3-oxazole based compound (**ML351**),³¹ purine-based (Anders-**6b**) compound,³² and pyrrole-based compound (**21B10**)³³ were

identified as 15-LOX-1 inhibitors as well (Figure 1). These inhibitors proved to be effective in various disease models, thus indicating the potential of 15-LOX-1 inhibitors for drug discovery.

Complementary to development of inhibitors, efforts were made to engineer 15-LOX-1 substrates for detection of enzyme activity. We developed activity-based probe **N144** as a chemical reporter for lipoxygenase activity in cell lysates and tissue samples.³⁴ Another study employed the omega-alkynyl fatty acid (**aAA**) to identify the intracellular targets of 12/15-LOX generated lipid-derived electrophiles.³⁵ This sets the stage for the development of potent 15-LOX-1 inhibitors and to study their cellular activity.

(A)



Figure 2. Examples of previously reported 15-LOX-1 inhibitors and chemical tools to study lipoxygenase activity. (A) Indole-based 15-LOX-1 inhibitor and inhibitors based on other nitrogen containing heterocycles. (B) Substrate-based chemical tools to study lipoxygenase activity in cell-based systems.

In this study, we investigated novel substitutions of the indole core and investigated the structure–activity relationships (SAR) for 15-LOX-1 inhibition. For the most potent inhibitor the effects on cellular 15-LOX-1 inhibition, the effects on formation of reactive oxygen species (ROS) and regulated cell death were investigated on RAW 264.7 macrophages to provide insight in the cellular potency of this type of inhibitors.

2. RESULTS AND DISCUSSION

2.1. Chemistry. Scheme 1 presents the general methodology for the synthesis of compounds **5a** and **5b**. The synthesis started with the assembly of ethyl 6-chloro-1*H*-indole-2-carboxylate (1) and corresponding aldehyde (2) using known literature procedures.^{30,36} Subsequently, the 2-formyl functionality of the aldehyde **2** was oxidized into its corresponding carboxylic acid (**3**) *via* Pinnick oxidation using sodium chlorite (NaClO₂) giving a yield of 78%. Attempts to use KMnO₄ or Tollens' reagent did not provide the desired product. Finally, the amide bonds in products **5a** and **5b** were generated by using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) and *N*-hydroxybenzotriazole (HOBt) as coupling reagents, giving yields of about 85%.



Scheme 1. Synthetic route to compounds 5a–b. Reagents and conditions: (a) POCl₃, DMF, 60 °C, 48 h; (b) NaClO₂, *t*-BuOH, 50 °C, 4 h; (c) amine (4a–b), EDCI, HOBT, TEA, DCM, r.t., 4 h.

Compounds **9a–j** were synthesized using procedures as shown in Scheme 2. As a first step the 2-formyl functionality of aldehyde **2** was employed for Wittig reaction with (*tert*butoxycarbonylmethylene) triphenylphosphorane to provide the α,β -unsaturated ester **6**. Initially, attempts were made to obtain compound **7** by refluxing aldehyde **2** with the Wittig reagent in toluene overnight. However, this provided compound **6** as a mixture of *E*- and *Z*-isomers (*E*/*Z* = approximately 9/1).³⁷ Changing the solvent from toluene to ethanol at 80 °C enabled the reaction to finish in 2 hours with the *E*-alkene as major product that could be isolated in a yield of 70% after purification. The *E*- and *Z*-isomer could be distinguished by their *J* values of 16.0 Hz and 7.0 Hz, respectively. Finally, intermediate **7** was converted into the amides **9a–j** by removal of the *tert*butyl protective group using trifluoroacetic acid (TFA) treatment and subsequent coupling of the corresponding amines using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) and *N*hydroxybenzotriazole (HOBt) as coupling reagents in yields between 80 and 90% over two steps.



Scheme 2. Synthetic route to compounds 9a–j. Reagents and conditions: (d) (*tert*-Butoxycarbonylmethylene) triphenylphosphorane, EtOH, reflux, 2 h; (e) TFA, DCM, r.t., overnight; (f) amine (8a–j), EDCI, HOBT, TEA, DCM, r.t., 4 h.

Products **12a–g** were produced starting from compound **10** as shown in Scheme 3. The carboxylic acids **10** was coupled to amines **11a–g** using EDCI and HOBT as coupling reagents, that gives the desired products in 80–90% yield. Afterwards, compound **13** was obtained from compound **12c** by using a Mannich reaction with dimethyl amine, formaldehyde and acetic acid to provide the desired product in a yield of 93%. As shown in Scheme 4, compounds **14–17** were obtained from the corresponding 2-carboxy ethyl indoles (**9c**, Eleftheriadis-**14d** and Eleftheriadis-**14e**) over two steps in the similar way as for **12a–g** in a high yield (80–90%).



Scheme 3. Synthetic route to compounds 12a–g and 13. Reagents and conditions: (g) amine (11a–g), EDCI, HOBT, TEA, DCM, r.t., 4 h; (h) dimethylamine, formaldehyde, acetic acid, MeOH, reflux, 4 h.



Scheme 4. Synthetic route to compounds 14–17. Reagents and conditions: (i) LiOH, THF, H₂O, 50 °C, 2 h; (j) EDCI, HOBT, TEA, DCM, r.t., 4 h.

2.2. Structure–activity relationships. Inhibition of 15-LOX-1 enzyme activity was performed using an activity assay as described previously by us.^{30,33,38} The activity of 15-LOX-1 was monitored by measuring the conversion of LA into the UV-active 13-HpODE (λ_{max} 234 nm). This assay was used to determine the half maximal inhibitory concentration (IC₅₀) of each

 compound. SARs for binding to 15-LOX-1 were investigated starting from ethyl 6-chloro-1*H*indole-2-carboxylate (1). We aimed to introduce structural modifications to replace the lipid chain in the 3-position and the ethylcarboxylate in the 2-position. Key structural modifications are shown in Figure 3 starting from previously identified inhibitors 1 and Eleftheriadis-14d.³⁰



Figure 3. Systematic modifications of 15-LOX-1 inhibitor 1 as core scaffold and the previously described inhibitor Eleftheriadis-14d³⁰ to provide the new inhibitors **5b**, **9c**, **15–17** and their IC₅₀ values for 15-LOX-1 inhibition.

The SAR of the previously identified inhibitor Eleftheriadis-14d, was explored with respect to aliphatic acyl substitutions in the indole 3-position. In order to expand the SAR in novel directions, the carboxyl ethyl ester in the indole 3-position was replaced by an amide in order to gain metabolic stability. Both compounds **5a** and **5b** (Scheme 1, Table 1) provided IC₅₀ values above 1 μ M, which is much higher compared to the previously reported series of inhibitors with the carboxyl ethyl ester.³⁰ Apparently, replacement of the carboxyl ethyl ester in the indole 2-position for an amide is unfavorable for 15-LOX-1 inhibition.



Table 1. IC₅₀ values for 15-LOX-1 inhibition by amide substituted indoles in the 3-position (analogues **5a–b**).

The SAR in the indole 3-position was further explored by replacement of the carbonyl in 3-position for a double bond using Wittig chemistry. Using this chemistry, we aimed to replace the aliphatic lipid chain in Eleftheriadis-14d for less flexible substituents. Thus, we investigated a series of *E*-alkenes as shown in Table 2. Clear SARs were observed for this series of compounds. Compound 9c (i472) proved to be the most potent 15-LOX-1 inhibitor with an IC₅₀ value of 0.19 μ M. Comparison of inhibitor 9c (i472) to inhibitor 9f indicates that the *ortho*-methoxy substitution on the benzyl provides a ten-fold gain in potency compared to a non-substituted benzyl. Importantly, the IC₅₀ values decrease if the methoxy is moved from the *ortho* to the *meta* or *para* position on the benzyl 9d and 9e. Extending the benzyl to an ethylphenyl in inhibitors 9g–j did not improve their potencies either. Taken together, the 2-methoxybenzyl group in 9c (i472) provides the most potent inhibitor in this series.

IC₅₀ (µM) R С Compound R ŃН $[\pm SD (\mu M)]$ CI CI 9a 2.95 ± 1.28 Ó C н 9b (i44) 0.74 ± 0.22 9c (i472) 0.19 ± 0.03 9d 0.40 ± 0.01 9e 0.75 ± 0.31 9f 2.43 ± 0.96 9g >20 CI 9h 1.29 ± 0.42 CI Cl 9i 0.19 ± 0.05 9j 0.27 ± 0.01



In order to further explore the SAR of previously identified inhibitor Eleftheriadis-14d, variations were made in the indolyl 2-position. Towards this aim, the ester group was replaced with various amides to provide inhibitors 12a–g as shown in Table 3. The results indicate that amide substitution provides inhibitors with potencies in the micromolar range. However, the SAR for modifications with methyl, ethyl, *n*-propyl, *n*-butyl, *n*-pentyl and branched alkyl groups proved to be relatively flat with potency differences of no more than two- to three-fold. Remarkably, cyclopropyl substitution in 12g turns out to be inactive ($IC_{50} > 20 \mu M$). Taken together, the investigated series of amide substituted indoles did not provide improved potency and the most potent inhibitor in this series is compound 12c with a propyl substitution.

Table 3. IC₅₀ values against 15-LOX-1 with different variations in the amide tail (analogues 12a–g).

| H N-R | Compound | D | $IC_{50}(\mu M)$ |
|----------|----------|--|-------------------|
| | Compound | K | $[\pm SD(\mu M)]$ |
| | 12a | ~~~ | 9.38 ± 2 .63 |
| | 12b | 32 | 6.88 ± 2.32 |
| | 12c | × | 4.09 ± 1.11 |
| | 12d | ×~~~~ | 13.02 ± 2.73 |
| | 12e | ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ | 10.94 ± 2.21 |
| | 12f | 2 Contraction of the second se | 9.82 ± 1.41 |
| | 12g | 2 | >20 |

As a next step, we combined ethyl- or propyl-substituted amides in the indole 2-position with substitutions in the 3-position to provide inhibitors **14–17** as shown in Table 4. Unfortunately,

combination of both modifications provided inhibitors with low potency. Apparently, the more polar amide bond is not well tolerated for enzyme inhibition and combination of modifications in the 2- and 3-position caused a greatly reduced potency. Inhibitor **13** also showed a complete loss in potency against 15-LOX-1. Taken together, we concluded that inhibitor **9c (i472)** has the highest potency of this series and that the IC_{50} value is in the same range as for the previously identified inhibitor Eleftheriadis-**14d**.

Table 4. IC₅₀ values against 15-LOX-1 with different variations in R¹ and R² (analogues 14–18).

| \mathbb{R}^1 H | Compound | R^1 | R ² | IC ₅₀ (μM) |
|------------------|----------|------------------|----------------|-----------------------|
| | F | | | $[\pm SD (\mu M)]$ |
| H O - | 13 | N I | × | >20 |
| | 14 | | × | >20 |
| | 15 | | × | >20 |
| | 16 | O N H | 2×2 | 5.81 ± 1.18 |
| | 17 | O Z N H | 2 | >20 |

2.3. Docking 15-LOX-1. In order to understand the observed SAR, a molecular modeling study was performed in which key inhibitors were docked in the 15-LOX-1 active site. In this study, docking was performed using Discovery studio (Dassault systèmes) version 2018. And the

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rabbit reticulocyte 15-LOX-1 crystal structure (PDB ID: 1LOX) was used for docking because of its high sequence similarity in the active site.³⁹ In this crystal structure, the ligand in the crystal structure was removed and the center of the binding sphere was set at the same position. Based on this position, CDocker, a CHARMm based method, was used and resulted in ten highest ranked poses for all selected inhibitors.

Based on the observed SAR, the most potent inhibitor **9c** (i472) was docked and compared to **9f** (lacking the methoxy group) and **16** (in which the ester is replaced for an amide). In both cases the potency decreased by at least ten-fold. The docking model suggested several interactions between the active site of 15-LOX-1 and **9c** (i472) as shown in Figure 4(A). Upon comparison of the docking of **9c** (i472) and **9f**, the 2-methoxy on the benzyl functionality provides two hydrogen bonds with GLN 548 and ILE 593, respectively (Figure 4(A)). This provides an explanation for the ten-fold potency difference between **9c** (i472) and **9f**. In addition, because of the hydrophobic character of 15-LOX-1, except from the edge of the active site, of the majority of the pocket is hydrophobic, as shown in brown in Figure 4 (B) and (C). The hydrophilic sites are shown in blue. Docking of compound **16**, in which an amide replaces the carboxy ethyl ester at the indole 2position, shows a positionally inverted orientation compared to **9c** (i472) and **9f** (Figure 4 (C) and S1 (C)). Apparently, the amide with an additional hydrogen bond donor does not fit in the same position as the ester in **9c** (i472). This change in orientation upon docking is also in line with the observed drop in potency for **16** compared to **9c** (i472) and **9f**.



Figure 4. Molecular modeling of selected compounds in the active site of 15-LOX-1 (PDB ID: 1LOX). The surface in the pocket is colored based on the relative hydrophobicity: brown for hydrophobic and blue for hydrophilic areas (A) Interactions of compound **9c (i472)** with the active site of the enzyme. (B) The preferred orientation of compound **9c (i472)** in the active site of the enzyme. (C) The preferred orientation of compound **16** in the active site of the enzyme that is opposite to **9c (i472)**.

2.4. Physicochemical properties of inhibitor 9c (i472). The α,β -unsaturated amide functionality in 9c (i472) is, as a Michael-acceptor, reactive towards conjugate addition by nucleophiles, such as thiols. In order to monitor this reactivity the UV spectrum of 9c (i472) was recorded upon incubation with 2-mercaptoethanol at pH 7.4. No changes in the UV spectrum were observed, which indicates that the chromophore, including the α,β -unsaturated system, did not change (Figure S2), thus indicating a reasonable stability of compound **9c** (i472) towards nucleophilic substitution. This stability might be attributed to the conjugation of the α , β -unsaturated double bond with the aromatic indole core.

Inhibitor **9c** (i472) has a calculated cLogP (ChemDraw Professional version 12.0) of 4.7, which is more than two order of magnitude lower compared to the previously identified inhibitor Eleftheriadis-14d (cLogP = 6.9), whereas both inhibitors have molecular weights around 400 g/mol. Considering the physicochemical properties, the newly identified inhibitor **9c** (i472) has fewer rotatable bonds and a cLogP that is more favorable for cellular permeability compared to the previously identified inhibitor Eleftheridis-14d.

2.5. LOX inhibitory potency of 9c (i472) in cells by activity-based labeling. As a next step in the characterization of inhibitor 9c (i472), the inhibition of cellular LOX activity was investigated. Towards this aim we employed a method for activity-based labeling of LOX activity in cell-based systems that we developed recently.³⁴ In this method, a covalent inhibitor of lipoxygenase activity is equipped with a terminal alkene for bioorthogonal labeling with biotin using the oxidative Heck reaction.⁴⁰ Here we employed this method to estimate the inhibitor treated and non-treated cell-lysates were exposed to covalent inhibitor N144 (Figure 2) for 2 minutes, and subsequently the samples were subjected to oxidative Heck reaction to link a biotinylated phenylboronic acid for detection. In parallel to the labeling the same samples were subjected to staining for β -actin as a loading control and antibody based detection of the amount of 15-LOX-1. Representative blots are shown in Figure 5. We observed a decreased intensity for the band for activity-based lipoxygenase labeling as detection by streptavidin-HRP. For comparison, the bands normalized with the β -actin antibody and the 15-LOX antibody were included as well, which show

 comparable intensities. Quantifications of the bands from three independent experiments are shown in Figure 6. From these results, we conclude that 15-LOX-1 inhibitor **9c(i472)** is able to inhibit the activity of cellular lipoxygenases.



Figure 5. Detection of the effect of inhibitor **9c (i472)** on the activity of 15-LOX-1 by activity-based probe. Labeling was performed on cell lysis of RAW 264.7 cells. Positive control (with probe and without inhibitor), negative control (without probe or inhibitor) and incubation of **9c (i472)** were performed with the 15-LOX antibody, β -actin antibody and streptavidin-HRP (*n*=3).



Figure 6. Quantification of Western blots for detection and analysis of activity-based labeling: The values are measured by integrating the gray values by ImageJ 1.44. The integrated gray values of streptavidin-HRP and 15-LOX are normalized to β -actin, respectively. All the values were expressed as means \pm SEM. The results were normalized by three independent experiments (*n*=3). *p < 0.05; **p < 0.005 and ***p < 0.001 compared to control by two-tailed test.

2.6. Protection of RAW 264.7 macrophages from LPS-induced cytotoxicity. After identifying compound 9c (i472) as a potent inhibitor for recombinant expressed 15-LOX-1 and cellular LOX activity, we moved on to investigate the potency of this compound in cell-based studies. As the insight into the mechanism, we presume that 15-LOX-1 inhibitors inhibit the formation of lipid peroxides in cells, thereby preventing ferroptotic cell death. Additionally, we expect this mechanism to have cross-talk with the NF- κ B pathway. Activation of this pathway can also lead to cell death. To test this hypothesis we employed a model in which we stimulated RAW264.7 macrophages with LPS to cause cell death as reported previously.³ This study reported an LD₅₀ of 89.5 µg/mL for LPS induced cell death in macrophages. In our experiments, 40% inhibition of cell viability was obtained at 100 µg/mL (Figure S3). Subsequently, as shown in

Figure 7, we employed an LPS concentration of 100 μ g/mL and investigated the protection from cell death by treatment with lipoxygenase inhibitors. The 5-LOX inhibitor Zileuton and the previously identified 15-LOX-1 inhibitor Eleftheriadis-14d comparably improved the viability of LPS-treated RAW 264.7 macrophages. In addition, inhibitor 9c (i472) showed stronger, dose-dependent effects with a 20% viability increase at 5 μ M. Thus, these data demonstrated that inhibiting 15-LOX-1 by compound 9c (i472) can protect RAW 264.7 macrophages from LPS-induced cell death.



Figure 7. Inhibitor **9c (i472)** protects RAW 264.7 macrophages from LPS-induced cytotoxicity. RAW 264.7 macrophages were treated with lipopolysaccharides (LPS) (100 μ g/mL) and **9c (i472)** together for 24 hours. Then, the cell viability was determined by MTS assay (*n*=3–13). All the values were expressed as means ± SEM. *p < 0.05; **p < 0.005 and ***p < 0.001 compared to positive control by two-tailed test that is only with the treatment of LPS (100 μ g/mL) for 48 hours.

2.7. NF- κ B activity determination. To gain further insight in the mechanism of protection for LPS-induced cell death, we investigated the effect of inhibitor 9c (i472) on NF- κ B activity

using an NF- κ B reporter assay in RAW-Blue macrophages (modified RAW 264.7 macrophages). These RAW-Blue macrophages can stably express a secreted embryonic alkaline phosphatase (SEAP) gene that is inducible by NF- κ B and AP-1 transcription factors. Previous evidence demonstrated that the product of 15-LOX-1, 13-HpODE, can increase NF- κ B activation but has no effect on AP-1.⁸ The cells were stimulated with LPS, interferon gamma (IFN γ) and inhibitor **9c** (i472).⁴¹ This provided significant but not complete inhibition of NF- κ B transcriptional activation upon LPS/INF γ stimulation (Figure 8). These results are in line with the anticipated cross-talk between 15-LOX-1 inhibition and NF- κ B signaling.



Figure 8. Inhibitor **9c (i472)** reduces NF- κ B activity. RAW-Blue macrophages were pre-treated with inhibitors **9c (i472)** at 0.2, 1 and 5 μ M for 20 hours, after which an inflammatory lipopolysaccharides (LPS) and interferon gamma (IFN γ) stimulus (10 ng/mL of each) was given for another 4 hours in continued presence of inhibitor **9c**. All the values were expressed as means ± SEM (*n*=8–16). *p < 0.05; **p < 0.005 and ***p < 0.001 compared to positive control that is treated with LPS and IFN γ by two-tailed test.

2.8. Gene Expression Profiling. Subsequently, we turned our attention to the influence of 15-LOX-1 inhibition by **9c (i472)** on the gene expression of NF-κB related gene iNOS (Figure 9).

As a model we used RAW 264.7 macrophages that were activated by LPS and IFN γ (10 ng/mL of each).⁴² The gene expression of iNOS was downregulated by approximately 50% at 5 μ M. This finding is in line with but more pronounced than the observed decrease in NF- κ B transcriptional activity (Figure 8).



Figure 9. Effect of inhibition of 15-LOX-1 by **9c (i472)** on iNOS in RAW 264.7 cells: Lipopolysaccharides (LPS)/ interferon gamma (IFN γ) (10 ng/mL of each) stimulated cells were normalized to the positive control. All experimental groups were treated with compound **9c (i472)** at 0.2, 1 and 5 μ M for 20 h and stimulated with LPS/IFN γ for another 4 hours (n=3–4). All the values were expressed as means ± SEM. *p < 0.05; **p < 0.005 and ***p < 0.001 compared to LPS/IFN γ treated positive control group by two-tailed test.

2.9. Quantification of Nitric oxide (NO) production. Gene transcription of iNOS is connected to NO production, which plays an important role in the regulation of immune response and apoptosis. In our study, we compared the inhibitory effects of **9c (i472)** on the ratio of total nitrate/nitrite in RAW 264.7 macrophages (Figure 10). We demonstrated that **9c (i472)** as 15-LOX-1 inhibitor provided dose-dependent inhibition of NO production, which is consistent with the results of reduced activity of NF- κ B and the gene expression of iNOS. The observations that 15-

LOX inhibition inhibits NF- κ B reporter gene activity, iNOS expression and NO levels are in line with the idea that there is cross-talk between 15-LOX-1 activity and cell-death *via* activity of the NF- κ B pathway and NO production.



Figure 10. Dose-dependent effect of **9c** (**i472**) on the expression of total nitrate/nitrite in RAW 264.7 cells: Lipopolysaccharides (LPS)/ interferon gamma (IFN γ) (10 ng/mL of each) stimulated cells were corrected to 100% as positive control. All experimental group were treated with compound **9c** (**i472**) at 0.2, 1 and 5 μ M for 20 h and stimulated with LPS/IFN γ for another 4 hours (n=3). All the values were expressed as means ± SEM. *p < 0.05; **p < 0.005 and ***p < 0.001 compared to LPS/IFN γ treated control group by two-tailed test.

2.10. Lipid peroxidation. Oxidative stress can cause a series of toxic effects through the production of lipid peroxides that play a role in cell death.⁴³ The effect of lipoxygenase inhibitor **9c (i472)** on lipid peroxidation RAW 264.7 cells was investigated using the fluorescent dye BODIPY 581/591 C₁₁ and fluorescence-activated cell sorting (FACS).⁴⁴ As shown in Figure 11, 15-LOX-inhibitor **PD-146176** and **9c (i472)** revealed comparable effects that both of them significantly attenuated the boost of lipid peroxides at 5 μ M after the treatment of LPS/IFN γ (10 ng/mL of each). This result could be attributed to loss of 15-LOX products, such as 13-HpODE.

Furthermore, compared to 5-LOX inhibitor, **Zileuton**, both 15-LOX-1 inhibitors showed a more pronounced effect in lipid peroxidation fitting the result of LPS-induced cell death assay that 15-LOX-1 inhibitor has a better rescue effect. Although 15-LOX-1 is not the only pathway that can trigger lipid peroxide formation, these results indicate that inhibition of 15-LOX-1 has a strong influence on lipid peroxidation in this model system.



Figure 11. Analysis lipid peroxidation using BODIPY 581/591 C11 staining and FACS analysis. Cells were treated with lipopolysaccharides (LPS)/ interferon gamma (IFN γ) (10 ng/mL of each) and **PD-146176** (5 μ M), **Zileuton** (5 μ M) or **9c (i472)** (5 μ M). Results are represented as means \pm SEM (n = 3). *p < 0.05; **p < 0.005 and ***p < 0.001 compared to LPS/IFN γ treated control group by two-tailed test.

3. Conclusions

In this study, compound 9c (i472) was developed as a potent 15-LOX-1 inhibitor with a novel substituent pattern (IC₅₀ = 0.19 μ M) and its SARs were explored. Using activity-based labeling we demonstrated that inhibitor 9c (i472) was able to inhibit cellular lipoxygenases. Further characterization of this compound demonstrated that it was able to protect RAW264.7

macrophages from LPS-induced cell death. We explored the influence of inhibitor 9c (i472) on different pathways of cell death. We investigated NF- κ B activation, iNOS expression and NO formation as a line of events that can trigger cell death. Treatment with inhibitor 9c (i472) enabled downregulation of NF- κ B transcriptional activity in a reporter gene assay. Furthermore, we demonstrated that iNOS gene expression and the levels NO in RAW 264.7 macrophages decreased significantly upon 9c (i472) treatment. As a direct effect of inhibiting lipoxygenase activity, we investigated inhibition of cellular lipid peroxidation upon 9c (i472) treatment for which we observed a clear reduction back to baseline levels. Having explored both mechanisms, we can conclude that inhibitor 9c (i472) influences both NO production and lipid peroxidation, potentially *via* a cross-talk mechanism. Thus, we conclude that we provide a novel 15-LOX-1 inhibitor 9c(i472) with cellular activity that inhibits the formation of oxidative mediators, such as NO and lipid peroxides, that are connected to different mechanisms for cell death.

4. Experimental section

4.1. General. All reagents, solvents and catalysts were purchased from commercial sources (Acros Organics, Sigma-Aldrich and abcr GmbH, Netherlands) and used without purification. All reactions were performed in oven-dried flasks in open or under nitrogen atmosphere, and monitored by thin layer chromatography on TLC precoated (250 μ m) silica gel 60 F254 aluminum foil (EMD Chemicals Inc.). Visualization was achieved using UV light. Alternatively, non UV-active compounds were detected after staining with potassium permanganate. Flash column chromatography was performed on silica gel (32-63 μ m, 60 Å pore size). ¹H NMR (500 MHz) and ¹³C NMR (126 MHz) spectra were recorded with a Bruker Avance 4-channel NMR Spectrometer with TXI probe. Chemical shifts (δ) are reported in ppm. Abbreviations are as follows: s (singlet),

d (doublet), t (triplet), q (quartet), m (multiplet). Fourier Transform Mass Spectrometry (FTMS) and electrospray ionization (ESI) were done on an Applied Biosystems/SCIEX API3000-triple quadrupole mass spectrometer. High performance liquid chromatography (HPLC) analysis was performed for confirming purity with a Shimadzu LC-10AT HPLC with a Shimadzu SP-M10A ELSD detector and a Shimadzu SPD-M10A photodiode array detector. Analytical HPLC was performed using a Kinetex C18 column (150 mm × 4.6 mm, 5 µm) with 5–95% MeCN gradient in H₂O as mobile phase, confirming purity \geq 95%. Retention time (RT) of HPLC was also reported.

4.2. Synthesis and Characterization.

4.2.1. Ethyl 6-chloro-3-formyl-1H-indole-2-carboxylate (2): To a solution of POCl₃ (0.30 mL, 3.2 mmol, 1.2 eq.) in DMF (6.0 mL) stirred at 0 °C for 0.5 h, **1** (0.60 g, 2.7 mmol), was added to the reaction mixture and heated to 50 °C for 46 h. After completion, the reaction mixture was slowly poured into a mixture of crushed ice and H₂O (300 mL). Product was obtained by filtration of the resulting suspension. The residue was washed with acetonitrile and dried at r.t. giving the title intermediate **2** as yellow solid in a yield of 83% (0.55 g, 2.2 mmol). The NMR spectra were the same as reported previously.³⁶

4.2.2. 6-chloro-2-(ethoxycarbonyl)-1H-indole-3-carboxylic acid (3): To a solution of 2 (0.10 g, 0.39 mmol) and NaClO₂ (71 mg, 0.78 mmol) mixed in 5.0 mL *t*-BuOH at 50 °C for 4h. After completion, the mixture was concentrated and H₂O (50 mL) was added, followed by extraction with EtOAc (3×15 mL). The organic phases were collected and evaporated giving a white crude product without further purification in 82% yield.

4.2.3. General synthetic procedure 1: Amide bond formation. The respective carboxylic acid (1.0 equiv.) was added to a mixture of HOBt (0.40 equiv.), EDCI (2.0 equiv.) and Et₃N (1.0

eq) in CH_2Cl_2 (20 mL) at room temperature for 30 minutes. After the stirring, the respective amine (1.5 equiv.) was added to this reaction mixture that was subsequently stirred at room temperature for 4 h. Then the reaction mixture was washed with 1.0 M aqueous HCl (5.0 mL), sat. aqueous NaHCO₃ (5.0 mL), brine (5.0 mL), dried over MgSO₄, filtered, and concentrated under reduced pressure. The crude product was purified by column chromatography eluted with 20% ethyl acetate in DCM as solvent to reach a white solid product with a general yield from 70% to 80%.

4.2.4. Ethyl 6-chloro-3-(propylcarbamoyl)-1H-indole-2-carboxylate (5a): The product was obtained using general procedure 1 starting from carboxylate **3** and propylamine. The product was obtained as yellow solid in a 86% yield. ¹H NMR (500 MHz, DMSO- d_6) δ 12.96 (s, 1H), 8.92 (t, J = 5.5 Hz, 1H), 8.57 (d, J = 2.5 Hz, 1H), 7.87 (d, J = 8.5 Hz, 1H), 7.35 (dd, J = 8.5, 2.5 Hz, 1H), 4.31 (q, J = 7.0 Hz, 2H), 3.26-3.23 (m, 2H), 1.59-1.52 (m, 2H), 1.32 (t, J = 7.0 Hz, 3H), 0.91 (t, J = 7.0 Hz, 3H). ¹³C NMR (126 MHz, DMSO- d_6) δ 167.37, 160.16, 154.97, 139.38, 136.90, 130.43, 130.37, 124.08, 120.02, 119.90, 63.31, 41.49, 22.49, 14.26, 11.88. HRMS, calculated for C₁₅H₁₈ClO₂N₃ [M + H]⁺: 309.1000, found 309.1002. HPLC: purity 96%, retention time 17.7 min.

4.2.5. Ethyl (R)-6-chloro-3-((2,6-dimethylheptyl)carbamoyl)-1H-indole-2-carboxylate (**5b**): The product was obtained using general procedure 1 starting from carboxylate **3**. The product was obtained as yellow solid in a 82% yield.¹H NMR (500 MHz, CDCl₃) δ 10.75 (s, 1H), 9.40 (s, 1H), 8.43 (d, *J* = 8.5 Hz, 1H), 7.50 (d, *J* = 2.0 Hz, 1H), 7.36 (dd, *J* = 8.5, 2.0 Hz, 1H), 5.75 (s, 1H), 4.56 (q, *J* = 7.0 Hz, 2H), 3.25-3.06 (m, 1H), 1.54 (m, 1H), 1.51 (t, *J* = 5.0 Hz, 3H), 1.36-1.15 (m, 8H), 0.96 (d, *J* = 6.0 Hz, 3H), 0.93 (d, *J* = 6.0 Hz, 3H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 166.71, 160.13, 154.91, 139.28, 136.81, 131.58, 130.40, 124.03, 120.33, 119.92, 63.29, 45.55, 38.70, 36.64, 36.34, 27.81, 24.04, 22.87, 20.86, 14.26, 14.18. HRMS, calculated for C₂₁H₃₀ClN₂O₃ [*M* + H]⁺: 393.1939, found 393.1938. HPLC: purity 99%, retention time 14.3 min.

4.2.6. Ethyl (E)-3-(3-(tert-butoxy)-3-oxoprop-1-en-1-yl)-6-chloro-1H-indole-2carboxylate (6): 2 (0.10 g, 0.39 mmol), (*tert*-butoxycarbonylmethylene) triphenylphosphorane (0.17 g, 0.50 mmol) and EtOH (10 mL) were mixed under an atmosphere of nitrogen in an ovendried flask. The mixture was heated at reflux for 2 h. Then the solvent was evaporated under reduced pressure. The product was purified by column chromatography eluted with 20% ethyl acetate in petroleum as solvent and obtained as a yellow solid in 84% yield. The NMR spectra were the same as reported previously.⁴⁵

4.2.7. (E)-3-(6-chloro-2-(ethoxycarbonyl)-1H-indol-3-yl)acrylic acid (7): **6** (0.50g, 1.7mmol) was dissolved in DCM (1mL). Then Trifluoroacetic acid (1.0 mL, 2.0 mmol) was added and the mixture was stirred at 0 °C for 2 h. After evaporation of the solvent, the crude product did not need further purification. The NMR spectra were the same as reported previously.⁴⁵

4.2.8. Ethyl (E)-6-chloro-3-(3-((2,4-dichlorobenzyl)amino)-3-oxoprop-1-en-1-yl)-1Hindole-2-carboxylate (9a): The product was obtained using general procedure 1 starting from 7. The product was obtained as white solid in a 89% yield.¹H NMR (500 MHz, DMSO- d_6) δ 12.31 (s, 1H), 8.63 (t, J = 6.0 Hz, 1H), 8.34 (d, J = 16.0 Hz, 1H), 8.06 (d, J = 8.5 Hz, 1H), 7.65 (d, J =2.0 Hz, 1H), 7.55 (d, J = 2.0 Hz, 1H), 7.48-7.43 (m, 2H), 7.28 (dd, J = 8.5, 2.0 Hz, 1H), 7.24 (d, J =16.0 Hz, 1H), 4.48 (d, J = 6.0 Hz, 2H), 4.41 (q, J = 7.0 Hz, 2H). 1.39 (t, J = 7.0 Hz, 3H). ¹³C NMR (126 MHz, DMSO- d_6) δ 166.16, 161.20, 137.57, 136.10, 133.64, 132.81, 132.02, 131.12, 130.47, 129.09, 127.88, 127.66, 123.97, 123.71, 122.58, 122.37, 117.17, 113.10, 105.90, 61.59, 14.69. HRMS, calculated for C₂₁H₁₈Cl₃N₂O₃ [M + H]⁺: 451.0378, found 451.0377. HPLC: purity 95%, retention time 19.2 min.

4.2.9. Ethyl (E)-6-chloro-3-(3-((2-chlorobenzyl)amino)-3-oxoprop-1-en-1-yl)-1Hindole-2-carboxylate (9b): The product was obtained using general procedure 1 starting from 7. The product was obtained as white solid in a 89% yield. ¹H NMR (500 MHz, DMSO- d_6) δ 12.32 (s, 1H), 8.59 (t, J = 5.5 Hz, 1H), 8.34 (d, J = 16.0 Hz, 1H), 8.07 (d, J = 6.0 Hz, 1H), 8.55 (d, J =2.0 Hz, 1H), 7.48 (dd, J = 7.0, 2.0 Hz, 1H), 7.43 (dd, J = 7.0, 2.0 Hz, 1H), 7.35 (m, 2H), 7.28 (dd, J = 7.0, 2.0 Hz, 1H), 7.03 (d, J = 16 Hz, 1H), 4.50 (d, J = 6.0 Hz, 2H), 4.42 (q, J = 6.0 Hz, 2H), 1.39 (t, J = 6.0 Hz, 3H). ¹³C NMR (126 MHz, DMSO- d_6) δ 171.36, 166.33, 141.59, 140.42, 134.65, 134.42, 133.89, 133.21, 131.51, 130.11, 129.20, 127.64, 126.67, 125.90, 122.91, 122.09, 117.00, 99.98, 65.72, 34.31, 19.24. HRMS, calculated for C₂₁H₁₉O₃N₂Cl₂ [M + H]⁺: 417.0767, found 417.0767. HPLC: purity 96%, retention time 19.2 min.

4.2.10. Ethyl (E)-6-chloro-3-(3-((2-methoxybenzyl)amino)-3-oxoprop-1-en-1-yl)-1Hindole-2-carboxylate (9c): The product was obtained using general procedure 1 starting from 7. The product was obtained as white solid in a 79% yield. ¹H NMR (500 MHz, DMSO- d_6) δ 12.28 (s, 1H), 8.39 (t, J = 5.5 Hz, 1H), 8.31 (d, J = 16.0, Hz, 1H), 8.08 (d, J = 9.0, Hz, 1H), 7.54 (dd, J =7.0, 2.0 Hz, 1H), 7.42-7.29 (m, 3H), 7.03 (s, 1H), 7.01 (d, J = 7.0 Hz, 1H), 6.94 (td, J = 7.0, 2.0 Hz, 1H), 4.43-4.38 (m, 4H), 3.83 (s, 3H), 1.39 (t, J = 7.0 Hz, 3H). ¹³C NMR (126 MHz, DMSO- d_6) δ 165.97, 161.25, 157.28, 141.43, 137.57, 131.43, 130.44, 128.78, 127.53, 125.63, 124.96, 124.11, 123.96, 122.30, 120.66, 117.37, 113.09, 111.04, 61.57, 55.78, 38.81, 14.73. HRMS, calculated for C₂₂H₂₂ClN₂O₄ [M + H]⁺: 413.1263, found 413.1261. HPLC: purity 96%, retention time 18.9 min.

4.2.11. Ethyl (E)-6-chloro-3-(3-((3-methoxybenzyl)amino)-3-oxoprop-1-en-1-yl)-1Hindole-2-carboxylate (9d): The product was obtained using general procedure 1 starting from 7. The product was obtained as white solid in a 91% yield. ¹H NMR (500 MHz, DMSO- d_6) δ 12.30 (s, 1H), 8.55 (t, J = 6.0 Hz, 1H), 8.34 (d, J = 16.0 Hz, 1H), 8.05 (d, J = 8.5 Hz, 1H), 7.55 (d, J = 2.0

Hz, 1H), 7.29-7.25 (m, 2H), 6.94 (d, J = 16.0 Hz, 1H), 6.91 (m, 2H), 6.85 (dd, J = 7.0, 2.0 Hz, 1H), 4.44-4.40 (m, 4H), 3.74 (s, 3H), 1.39 (t, J = 7.0 Hz, 3H). ¹³C NMR (126 MHz, DMSO- d_6) δ 165.92, 161.24, 159.82, 141.43, 137.57, 131.69, 130.45, 129.68, 127.53, 125.63, 124.96, 123.97, 123.05, 122.34, 120.13, 117.30, 113.66, 112.71, 61.58, 55.54, 42.84, 14.75. HRMS, calculated for $C_{22}H_{22}ClN_2O_4 [M + H]^+$: 413.1263, found 413.1262. HPLC: purity 96%, retention time 14.4 min.

4.2.12. Ethyl (E)-6-chloro-3-(3-((4-methoxybenzyl)amino)-3-oxoprop-1-en-1-yl)-1Hindole-2-carboxylate (9e): The product was obtained using general procedure 1 starting from 7. The product was obtained as white solid in 84% yield. ¹H NMR (500 MHz, DMSO- d_6) δ 12.29 (s, 1H), 8.49 (t, J = 6.0 Hz, 1H), 8.34 (d, J = 16.0 Hz, 1H), 8.04 (d, J = 6.0 Hz, 1H), 7.54 (d, J = 2.0Hz, 1H), 7.26 (m, 3H),6.97-6.91 (m, 3H), 4.43 (q, J = 6.0 Hz, 2H), 4.35 (d, J = 6.0 Hz, 2H), 3.74 (s, 3H), 1.39 (t, J = 6.0 Hz, 3H). ¹³C NMR (126 MHz, DMSO- d_6) δ 165.77, 161.24, 158.78, 137.56, 131.76, 131.52, 130.43, (2 ×) 129.35, 127.48, 123.95, 123.72, 123.22, 122.32, 117.33, 114.27, 114.21, 113.11, 61.58, 55.52, 42.35, 14.67. HRMS, calculated for C₂₂H₂₂C₁N₂O₄ [M + H]⁺: 413.1263, found 413.1262. HPLC: purity 95%, retention time 14.4 min.

4.2.13. Ethyl (E)-3-(3-(benzylamino)-3-oxoprop-1-en-1-yl)-6-chloro-1H-indole-2carboxylate (9f): The product was obtained using general procedure 1 starting from 7. The product was obtained as white solid in a 88% yield. ¹H NMR (500 MHz, DMSO- d_6) δ 12.30 (s, 1H), 8.57 (t, J = 6.0 Hz, 1H), 8.33 (d, J = 16.0 Hz, 1H), 8.05 (d, J = 6.0 Hz, 1H), 7.55 (d, J = 2.0 Hz, 1H), 7.38-7.25 (m, 6H), 6.67 (d, J = 16.0 Hz, 1H), 4.44-4,40 (m, 4H), 1.38 (t, J = 6.0 Hz, 3H). ¹³C NMR (126 MHz, DMSO- d_6) δ 165.91, 161.24, 139.86, 137.56, 131.68, 131.62, 130.44, 128.86, (2 ×) 127.98, 127.52, 127.38, 123.96, 123.77, 123.08, 122.34, 117.30, 113.02, 61.59, 42.88, 14.76.

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HRMS, calculated for $C_{21}H_{20}ClN_2O_3$ [*M* + H]⁺: 383.1157, found 383.1158. HPLC: purity 98%, retention time 7.1 min.

4.2.14. Ethyl (E)-6-chloro-3-(3-oxo-3-(phenethylamino)prop-1-en-1-yl)-1H-indole-2carboxylate (9g): The product was obtained using general procedure 1 starting from 7. The product was obtained as white solid in a 76% yield. ¹H NMR (500 MHz, DMSO- d_6) δ 12.28 (s, 1H), 8.29 (d, J = 16.0 Hz, 1H), 8.19 (t, J = 6.0 Hz, 1H), 8.02 (d, J = 7.5 Hz, 1H), 7.54 (d, J = 2.0 Hz, 1H), 7.34-7.21 (m, 6H), 6.90 (d, J = 16.0 Hz, 1H), 4.42 (q, J = 6.0 Hz, 2H), 4.45 (q, J = 6.0 Hz, 2H), 2.81 (t, J = 6.0 Hz, 2H), 1.38 (t, J = 6.0 Hz, 3H). ¹³C NMR (126 MHz, DMSO- d_6) δ 165.90, 161.24, 144.40, 139.98, 137.58, 137.13, 131.26, 130.41, (2 ×) 129.09, 128.82, 126.57, 123.97, 123.70, 123.26, 122.28, 117.34, 113.06, 61.55, 39.77, 35.74, 14.71. HRMS, calculated for C₂₂H₂₂ClN₂O₃ [M + H]⁺: 397.1313, found 397.1311. HPLC: purity 96%, retention time 14.5 min.

4.2.15. Ethyl (E)-6-chloro-3-(3-((2-chlorophenethyl)amino)-3-oxoprop-1-en-1-yl)-1Hindole-2-carboxylate (9h): The product was obtained using general procedure 1 starting from 7. The product was obtained as white solid in a 90% yield. ¹H NMR (500 MHz, DMSO- d_6) δ 12.28 (s, 1H), 8.28 (d, J = 16.0 Hz, 1H), 8.24 (t, J = 6.0 Hz, 1H), 8.02 (d, J = 9.0 Hz, 1H), 7.55 (d, J = 2.0 Hz, 1H), 7.46 (dd, J = 8.0, 2.0 Hz, 1H), 7.38 (dd, J = 8.0, 2.0 Hz, 1H), 7.33-7.26 (m, 3H), 6.87 (d, J = 16.0 Hz, 1H), 4.42 (q, J = 7.0 Hz, 2H), 3.45 (q, J = 7.0 Hz, 2H), 2.95 (t, J = 7.0 Hz, 2H), 1.40 (t, J = 7.0 Hz, 3H). ¹³C NMR (126 MHz, DMSO- d_6) δ 166.01, 161.53, 137.21, 136.78, 133.50, 131.21, 129.66, 129.44, 128.67, 128.58, 127.78, 127.68, 125.82, 125.02, 123.80, 120.88, 118.46, 112.26, 61.13, 38.63, 33.14, 14.71. HRMS, calculated for C₂₂H₂₁O₃N₂Cl₂ [M + H]⁺: 431.0924, found 431.0924. HPLC: purity 95%, retention time 14.2 min.

4.2.16. Ethyl (E)-6-chloro-3-(3-((2,4-dichlorophenethyl)amino)-3-oxoprop-1-en-1-yl)-1H-indole-2-carboxylate (9i): The product was obtained using general procedure 1 starting from 7. The product was obtained as white solid in a 89% yield. ¹H NMR (500 MHz, DMSO- d_6) δ 12.29 (s, 1H), 8.28 (d, J = 16.0 Hz, 1H), 8.22 (t, J = 6.0 Hz, 1H), 8.01 (d, J = 9.0 Hz, 1H), 7.62 (d, J =2.0 Hz, 1H), 7.55 (d, J = 2.0 Hz, 1H), 7.40 (m, 2H), 7.28 (m, 1H), 6.85 (d, J = 16.0 Hz, 1H), 4.42 (q, J = 7.0 Hz, 2H), 3.48 (q, J = 7.0 Hz, 2H), 2.91 (t, J = 7.0 Hz, 2H), 1.36 (t, J = 7.0 Hz, 3H). ¹³C NMR (126 MHz, DMSO- d_6) δ 165.97, 161.22, 137.55, 136.49, 134.57, 132.79, 132.21, 131.38, 130.44, 129.12, 127.86, 127.49, 123.97, 123.65, 123.13, 122.32, 117.29, 113.07, 61.56, 38.69, 32.96, 14.70. HRMS, calculated for C₂₂H₂₀O₃N₂Cl₃ [M + H]⁺: 465.0534, found 465.0534. HPLC: purity 95%, retention time 18.2 min.

4.2.17. Ethyl (E)-6-chloro-3-(3-((2-methoxyphenethyl)amino)-3-oxoprop-1-en-1-yl)-1H-indole-2-carboxylate (9j): The product was obtained using general procedure 1 starting from 7. The product was obtained as white solid in a 81% yield. ¹H NMR (500 MHz, DMSO- d_6) δ 12.27 (s, 1H), 8.27 (d, J = 16.0 Hz, 1H), 8.17 (t, J = 5.5 Hz, 1H), 8.02 (d, J = 8.5 Hz, 1H), 7.54 (d, J =2.0 Hz, 1H), 7.27 (dd, J = 8.0, 2.0 Hz, 1H), 7.22 (m, 1H), 7.18 (dd, J = 7.0, 2.0 Hz, 1H), 6.99 (d, J =16.0 Hz, 1H), 6.89 (m, 2H), 4.42 (q, J = 7.0 Hz, 2H), 3.81 (s, 3H), 3.32 (q, J = 7.0 Hz, 2H), 2.78 (t, J = 7.0 Hz, 2H), 1.39 (t, J = 7.0 Hz, 3H). ¹³C NMR (126 MHz, DMSO- d_6) δ 165.84, 161.25, 157.71, 137.56, 131.17, 130.43, 128.09, 127.61, 127.41, 123.96, 123.75, 123.68, 123.41, 122.31, 117.38, 113.11, 113.00, 111.12, 61.56, 55.80, 55.71, 30.50, 14.75. HRMS, calculated for C₂₃H₂₄O₄N₂Cl [M + H]⁺: 427.1419, found 427.1418. HPLC: purity 96%, retention time 17.2 min.

4.2.18. 6-chloro-N-methyl-1H-indole-2-carboxamide (12a): The product was obtained using general procedure 1 starting from **10**. The product was obtained as light brown solid in a 92% yield. ¹H NMR (500 MHz, DMSO- d_6) δ 11.72 (s, 1H), 8.53 (d, J = 5.0 Hz, 1H), 7.65 (d, J = 8.5

Hz, 1H), 7.43 (d, J = 2.0 Hz, 1H), 7.09 (d, J = 2.0 Hz, 1H), 7.05 (dd, J = 8.5, 2.0 Hz, 1H), 2.82 (d, J = 4.5 Hz, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 172.08, 162.03, 143.15, 135.19, 125.34, 125.14, 122.83, 113.47, 99.98, 35.69. HRMS, calculated for C₁₀H₁₀ClN₂O [M + H]⁺: 209.0476, found 209.0475. HPLC: purity 95%, retention time 18.5 min.

4.2.19. 6-chloro-N-ethyl-1H-indole-2-carboxamide (12b): The product was obtained using general procedure 1 starting from **10**. The product was obtained as light brown solid in a 94% yield. ¹H NMR (500 MHz, DMSO- d_6) δ 11.70 (s, 1H), 8.65 (t, J = 6.0 Hz, 1H), 7.69 (d, J = 8.5 Hz, 1H), 7.43 (d, J = 2.0 Hz, 1H), 7.12 (d, J = 2.0 Hz, 1H), 7.06 (dd, J = 8.5, 2.0 Hz, 1H), 3.31 (m, 2H), 1.15 (t, J = 7.5 Hz, 3H). ¹³C NMR (126 MHz, DMSO- d_6) δ 160.95, 137.05, 133.44, 128.15, 126.35, 123.55, 120.62, 112.17, 102.68, 34.13, 15.38. HRMS, calculated for C₁₁H₁₂ON₂Cl [M + H]⁺: 223.0633, found 223.0633. HPLC: purity 98%, retention time 10.8 min.

4.2.20. 6-chloro-N-propyl-1H-indole-2-carboxamide (12c): The product was obtained using general procedure 1 starting from **10**. The product was obtained as light brown solid in a 91% yield. ¹H NMR (500 MHz, DMSO-*d*₆) δ 11.70 (s, 1H), 8.56 (t, *J* = 5.5 Hz, 1H), 7.64 (d, *J* = 8.5 Hz, 1H), 7.45 (d, *J* = 2.0 Hz, 1H), 7.15 (d, *J* = 2.0 Hz, 1H), 7.05 (dd, *J* = 8.5, 2.0 Hz, 1H), 3.25 (q, *J* = 7.0 Hz, 2H), 1.55 (q, *J* = 7.5 Hz, 2H), 0.91 (d, *J* = 2.0 Hz, 1H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 161.14, 137.07, 133.42, 128.18, 126.36, 123.54, 120.57, 112.18, 102.75, 41.06, 22.97, 11.94. HRMS, calculated for C₁₂H₁₄ON₂Cl [*M* + H]⁺: 237.0789, found 237.0789. HPLC: purity 95%, retention time 14.5 min.

4.2.21. N-butyl-6-chloro-1H-indole-2-carboxamide (12d): The product was obtained using general procedure 1 starting from 10. The product was obtained as light brown solid in a 90% yield. ¹H NMR (500 MHz, DMSO- d_6) δ 11.96 (s, 1H), 7.45 (t, J = 6.0 Hz, 1H), 6.64 (d, J = 8.5 Hz,

1H), 7.43 (d, J = 2.0 Hz, 1H), 7.14 (d, J = 2.0 Hz, 1H), 7.20 (dd, J = 8.5, 2.0 Hz, 1H), 3.32 (q, J = 7.5 Hz, 2H), 1.57-1.53 (m, 2H), 1.39-1.34 (m, 2H), 0.90 (t, J = 7.5 Hz, 3H). ¹³C NMR (126 MHz, DMSO- d_6) δ 161.07, 137.05, 133.42, 128.15, 126.36, 123.57, 120.59, 112.13, 102.70, 38.93, 31.79, 20.10, 14.20. HRMS, calculated for C₁₃H₁₆ClN₂O [M + H]⁺: 251.0946, found 251. 0947. HPLC: purity 99%, retention time 14.5 min.

4.2.22. 6-chloro-N-pentyl-1H-indole-2-carboxamide (12e): The product was obtained using general procedure 1 starting from **10**. The product was obtained as light brown solid in a 91% yield. ¹H NMR (500 MHz, DMSO- d_6) δ ¹H NMR (500 MHz, DMSO- d_6) δ : 11.69 (s, 1H), 7.51 (t, J = 6.0 Hz, 1H), 6.66 (d, J = 8.5 Hz, 1H), 7.43 (s, 1H), 7.14 (m, 1H), 7.03 (dd, J = 8.5, 2.0 Hz, 1H), 3.30 (q, J = 7.5 Hz, 2H), 1.57-1.53 (m, 2H), 1.24-1.20 (m, 4H), 0.92 (t, J = 7.5 Hz, 3H). ¹³C NMR (126 MHz, DMSO- d_6) δ 161.05, 137.05, 133.42, 128.15, 126.35, 123.54, 120.55, 112.15, 102.72, 39.21, 29.36, 29.15, 22.36, 14.44. HRMS, calculated for C₁₄H₁₈ClN₂O [M + H]⁺: 265.1102, found 265.1103. HPLC: purity 99%, retention time 14.3 min.

4.2.23. 6-chloro-N-isopropyl-1H-indole-2-carboxamide (12f): The product was obtained using general procedure 1 starting from **10**. The product was obtained as light brown solid in a 84% yield. ¹H NMR (500 MHz, DMSO- d_6) δ ¹H NMR (500 MHz, DMSO- d_6) δ : 11.67 (s, 1H), 8.30 (d, J = 8.0 Hz, 1H), 6.66 (d, J = 8.5 Hz, 1H), 7.43 (s, 1H), 7.14 (d, J = 2.0 Hz, 1H), 7.03 (dd, J = 8.5, 2.0 Hz, 1H), 4.17-4.09 (m, 1H), 1.20 (d, J = 6.5 Hz, 6H) . ¹³C NMR (126 MHz, DMSO- d_6) δ 160.28, 137.04, 133.50, 128.14, 126.33, 123.46, 120.56, 112.10, 102.88, 41.24, 41.14, 22.87. HRMS, calculated for C₁₂H₁₄ON₂Cl [M + H]⁺: 237.0789, found 237.0789. HPLC: purity 99%, retention time 10.8 min.

4.2.24. 6-chloro-N-cyclopropyl-1H-indole-2-carboxamide (12g): The product was obtained using general procedure 1 starting from 10. The product was obtained as light brown solid in a 91% yield. ¹H NMR (500 MHz, DMSO- d_6) δ 11.70 (s, 1H), 8.54 (d, J = 2.0 Hz, 1H), 6.66 (d, J = 8.5 Hz, 1H), 7.43 (d, J = 2.0 Hz 1H), 7.12 (d, J = 2.0 Hz, 1H), 7.06 (dd, J = 8.5, 2.5 Hz, 1H), 2.90-2.83 (m, 1H). 0.75-0.71 (m, 2H), 0.61-0.55 (m, 2H). ¹³C NMR (126 MHz, DMSO- d_6) δ 162.36, 137.09, 133.14, 128.24, 126.32, 123.54, 120.63, 112.16, 103.01, 23.12, (2 ×) 6.30. HRMS, calculated for C₁₂H₁₂ON₂Cl [M + H]⁺: 235.0633, found 235.0632. HPLC: purity 97%, retention time 11.8 min.

4.2.25. 6-chloro-3-((dimethylamino)methyl)-N-propyl-1H-indole-2-carboxamide (13): 6-chloro-*N*-propyl-1*H*-indole-2-carboxamide (12c) (50 mg, 0.20 mmol), dimethylamine (9.5 mg, 0.20 mmol), paraformaldehyde (6.5 mg, 0.20 mmol) and 0.20 mL acetic acid were dissolved in 10 mL MeOH. The reaction mixture was refluxed for 4 h. After completion, the product was purified by column chromatography eluted with 10% ethyl acetate in petroleum ether as solvent to obtain a white solid product with a 91% yield. ¹H NMR (500 MHz, DMSO-*d*₆) δ 11.75 (s, 1H), 10.53 (t, *J* = 2.0 Hz, 1H), 7.71 (d, *J* = 8.5 Hz, 1H), 7.43 (d, *J* = 2.0 Hz, 1H), 7.07 (dd, *J* = 8.5, 2.0 Hz, 1H), 3.69 (s, 2H), 3.29 (q, *J* = 6.5 Hz, 2H), 2.24 (s, 6H), 1.71-1.66 (m, 2H), 1.04 (t, *J* = 7.0 Hz, 3H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 161.68, 135.54, 132.22, 128.27, 127.28, 121.36, 120.42, 112.11, 112.05, 52.57, 44.00, 43.97, 41.30, 22.75, 12.00. HRMS, calculated for C₁₅H₂₁ON₃Cl [*M* + H]⁺: 294.1368, found 294.1367. HPLC: purity 99%, retention time 13.9 min.

4.2.26. General synthetic procedure 2: hydrolysis reaction. Ester (1.0 equiv.) was dissolved in THF (15 mL) while stirring. Then a solution of lithium hydroxide trihydrate (3.0 equiv.) in demiwater (15 mL) was added and the mixture was stirred at 50 °C for 2 h. Subsequently, the

aqueous layer was extracted with EtOAc (3×15 mL). The combined organic layers were washed with brine, dried over MgSO₄, filtered, and concentrated under reduced pressure. The crude product **11** was used without further purification.

4.2.27. (**R**)-6-chloro-3-(3,7-dimethyloctanoyl)-N-propyl-1H-indole-2-carboxamide (14): The product was obtained using general procedure 1 and 2 starting from compound Eleftheriaids-14d and 12c. The product was obtained as light brown solid in a 65% yield over two steps. ¹H NMR (500 MHz, CDCl₃) δ 11.83 (s, 1H), 11.45 (t, *J* = 2.0 Hz, 1H), 7.88 (d, *J* = 8.5 Hz, 1H), 7.33 (d, *J* = 2.0 Hz, 1H), 7.31 (dd, *J* = 8.5, 2.5 Hz), 3.62 (q, *J* = 6.5 Hz, 2H), 3.06 (m, 2H), 2.28 (m, 1H), 1.83 (m, 2H), 1.56 (m, 1H), 1.42 (m, 2H), 1.33 (m, 2H), 1.22 (m, 2H), 1.12 (t, *J* = 7.5 Hz, 3H), 1.04 (d, *J* = 7.5 Hz, 3H), 0.89 (d, *J* = 7.5 Hz, 6H). ¹³C NMR (126 MHz, CDCl₃) δ 199.61, 159.84, 136.61, 135.02, 130.45, 125.26, 123.66, 123.16, 115.54, 113.22, 51.18, 42.02, 39.08, 37.33, 29.61, 27.92, 24.79, 22.69, 22.62, 22.56, 20.01, 11.66. HRMS, calculated for C₂₂H₃₂O₂N₂Cl [*M* + H]⁺: 391.2147, found 391.2148. HPLC: purity 97%, retention time 21.4 min.

4.2.28. (S)-6-chloro-3-(3,7-dimethyloctanoyl)-N-propyl-1H-indole-2-carboxamide (15): The product was obtained using general procedure 1 and 2 starting from compound Eleftheriaids-14e and 12c. The product was obtained as light brown solid in a 55% yield over two steps. ¹H NMR (500 MHz, CDCl₃) δ 11.63 (s, 1H), 11.41 (t, J = 2.0 Hz, 1H), 7.88 (d, J = 8.5 Hz, 1H), 7.71 (d, J = 2.0 Hz, 1H), 7.31 (dd, J = 8.5, 2.5 Hz), 3.60 (q, J = 7.0 Hz, 2H), 3.19-2.96 (m, 2H), 2.30-2.25 (m, 1H), 1.85-1.78 (m, 2H), 1.60-1.52 (m, 1H), 1.46-1.16 (m, 6H), 1.12 (t, J = 7.5Hz, 3H), 1.04 (d, J = 6.5 Hz, 3H), 0.90 (d, J = 6.5 Hz, 6H). ¹³C NMR (126 MHz, CDCl₃) δ 199.60, 159.81, 136.62, 134.97, 130.46, 125.27, 123.66, 123.17, 115.53, 113.18, 51.18, 42.00, 39.08, 37.32, 29.60, 27.92, 24.79, 22.69, 22.62, 22.56, 20.00, 11.66. HRMS, calculated for C₂₂H₃₂O₂N₂Cl [M + H]⁺: 391.2147, found 391.2149. HPLC: purity 99%, retention time 22.9 min.

4.2.29. (E)-6-chloro-N-ethyl-3-(3-((2-methoxybenzyl)amino)-3-oxoprop-1-en-1-yl)-1H-indole-2-carboxamide (16): The product was obtained using general procedure 1 and 2 starting from compound **9c** and **11b**. The product was obtained as white solid in a 62% yield over two steps. ¹H NMR (500 MHz, DMSO- d_6) δ 12.01 (s, 1H), 8.47 (t, J = 6.0 Hz, 1H), 8.31 (t, J = 6.0 Hz, 1H), 8.19 (d, J = 9.0 Hz, 1H), 8.02 (d, J = 7.5 Hz, 1H), 7.50 (s, 1H), 7.25 (m, 3H), 7.04 (d, J = 7.5 Hz, 1H), 6.92 (t, J = 7.5 Hz, 1H), 6.89 (d, J = 9.0 Hz, 1H), 4.38 (d, J = 6.0 Hz, 2H), 3.84 (s, 3H), 3.33 (m, 2H), 1.19 (t, J = 6.0 Hz, 3H). ¹³C NMR (126 MHz, DMSO- d_6) δ 166.34, 161.31, 157.29, 136.87, 134.45, 132.09, 129.02, 128.73, 127.32, 124.16, 123.14, 121.79, 120.79, 120.63, 113.16, 112.59, 111.05, 55.85, 37.93, 34.56, 31.15, 15.04. HRMS, calculated for C₂₂H₂₃ClN₃O₃ [M + H]⁺: 412.4122, found 412.4123. HPLC: purity 96%, retention time 13.8 min.

4.2.30. (E)-6-chloro-3-(3-((2-methoxybenzyl)amino)-3-oxoprop-1-en-1-yl)-N-propyl-1H-indole-2-carboxamide (17): The product was obtained using general procedure 1 and 2 starting from compound **9c** and **11c**. The product was obtained as white solid in a 62% yield over two steps. ¹H NMR (500 MHz, DMSO-*d*₆) δ 12.03 (s, 1H), 8.47 (t, *J* = 5.5 Hz, 1H), 8.29 (t, *J* = 5.5 Hz, 1H), 8.07 (d, *J* = 16.0 Hz, 1H), 8.01 (d, *J* = 9.0 Hz, 1H), 7.52 (d, *J* = 2.0 Hz, 1H), 7.23 (m, 3H), 7.20 (dd, *J* = 7.5, 2.0 Hz, 1H), 6.94 (td, *J* = 6.5, 2.0 Hz, 1H), 6.90 (d, *J* = 16.0 Hz, 1H), 4.38 (d, *J* = 6.0 Hz, 2H), 3.86 (s, 3H), 3.28 (q, *J* = 6.0 Hz, 2H), 1.68 (m, 2H), 0.95 (t, *J* = 6.0 Hz, 3H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 166.32, 161.45, 157.26, 136.87, 134.57, 132.07, 128.98, 128.69, 128.66, 127.30, 124.13, 123.12, 121.77, 120.73, 120.62, 113.06, 112.58, 111.03, 55.84, 41.44, 37.92, 22.72, 12.01. HRMS, calculated for C₂₃H₂₅ClN₃O₃ [*M* + H]⁺: 426.1579, found 426,1580. HPLC: purity 96%, retention time 13.8 min.

4.3. Human 15-LOX-1 enzyme inhibition studies. The 15-LOX-1 enzyme was expressed and purified as described before.⁴⁶ Furthermore, the 15-LOX-1 enzyme activity studies were done

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using procedures previously described by our group as well.³⁰ 15-LOX-1 activity was determined by the conversion of linoleic acid to hydroperoxy-9*Z*,11*E*-octadecadienoic acid (λ_{max} of 234 nm) in a 96-well plate. The conversion rate was followed by UV absorbance at 234 nm. The conversion rate was evaluated at the linear part of the plot, and the substrate depletion covers the first 16 min. The optimum concentration of 15-LOX-1 was determined by an enzyme activity assay and proved to be a 40-fold dilution. The assay buffer consists of 25 mM HEPES titrated to pH 7.4. The substrate, linoleic acid (LA) (Sigma-Aldrich, L1376), was dissolved in ethanol to a concentration of 500 nM. The absorbance increased at 234 nm over time for the conversion of linoleic acid in the presence (positive control) of the enzyme, or remained constant in the absence (blank control) of the enzyme.

To determine IC₅₀ values, 140 μ L of the inhibitors (0 – 71 μ M, 2 × dilution series) were incubated with 50 μ L of 1:40 enzyme solution for 10 min at room temperature in 96-well plate. After 10 min incubation, 10 μ L of 500 nM LA was added to the mixture, which resulted in desired concentrations of the inhibitors (0 – 50 μ M, 2 × dilution series), a final dilution of the enzyme of 1:160 and 25 nM LA. The linear absorbance increased in the absence of the inhibitor was set to 100%, whereas the absorbance increased in the absence of the enzyme was set to 0%. All experiments were performed at least in triplicate. The average values and their standard deviations were plotted. Data analysis was performed using Microsoft Excel professional plus 2013 and GraphPad Prism 5.01.

4.4. Cell culture, MTS and RAW-Blue NF-\kappaB reporter gene assay. RAW 264.7 murine macrophages were obtained from ATCC (Wesel, Germany) and cultured in Dulbecco Modified Eagle Medium + Glutamax (Gibco by Life Technologies, The Netherlands) supplemented with 10% (v/v) Fetal Bovine Serum and 100 U/mL 1% penicillin/streptomycin (Gibco, The Netherlands) in a humidified 5% CO₂ atmosphere at 37 °C. RAW-Blue macrophages were obtained from $_{39}$

InvivoGen (Toulouse, France) and cultured in the same conditions, with the addition of 200 μ g/mL Zeocin to the culture medium as reported by the manufacturer.

RAW 264.7 cells were seeded at 5,000 cells per well in a 96 well plate one day prior to the experiment. Cells were treated with **9c (i472)** at 0.1, 1, 5, 10 and 50 μ M for 48 h. The cell viability of the treated cells was determined by CellTiter reagent that 20 μ L CellTiter reagent was added to each well. After 2 h incubation with the CelTiter reagent at 37 °C, the absorbance at 490 nm was measured using a Synergy H1 plate reader.

RAW-Blue cells were seeded at 10×10^4 cells per well in a 96 well plate one day before the start of the experiment. Cells were treated with **9c (i472)** at 0.2, 1 and 5 µM and stimulated with 10 ng/mL LPS (Sigma-Aldrich, The Netherlands) and 10 ng/mL IFN γ (Sigma-Aldrich, The Netherlands) for 24h. The secreted embryonic alkaline phosphatase (SEAP) release was measured to monitor the NF- κ B levels using the QuantiBlue reagent (InvivoGen, Toulouse, France). After 2h incubation at 37 °C, the absorbance at 635-655 nm was measured using Synergy H1 plate reader according to the manufacturer's instructions.

4.5. LPS induced cell death. RAW 264.7 cells were seeded at 5,000 cells per well in a 96well plate. The ability of rescue were tested with the treatment of **9c (i472)** at 0.2, 1 and 5 μ M or Zileuton at 5 μ M with 100 μ g/mL LPS for 48 h. The cell viability was determined by MTS assay as described above.³

4.6. Gene Expression Analysis by RT-qPCR. Total RNA was isolated from RAW 264.7 cells using the SV total RNA isolation system (Promega, Leiden, The Netherlands) and performed according to the protocol of the manufacturer. RNA integrity was determined by 28S/18S ratio detection on an agarose gel, which was consistently found to be intact. For gene expression analysis,

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RNA was reverse-transcribed using a reverse-transcription kit (Promega). Subsequently, 10 ng of cDNA was applied for each real-time PCR, which was performed on an ABI Prism 7900HT sequence detection system (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The primers for NF-ĸB1 (Fw, 5'- GAAATTCCTGATCCAGACAAAAAC-3', Rv, 5'-ATCACTTCAATGGCCTCTGTGTAG-3'), 5'-NF-_KB2 (Fw. CTGGTGGACACATACAGGAAGAC-3', Rv, 5'- ATAGGCACTGTCTTCTTCACCTC-3'), RelA 5'-(Fw, CTTCCTCAGCCATGGTACCTCT-3', Rv. 5'-CAAGTCTTCATCAGCATCAAACTG -3'), RelB (Fw, 5'- CTTTGCCTATGATCCTTCTGC-3', 5'-GAGTCCAGTGATAGGGGGCTCT-3') and iNOS (Fw, 5'-Rv. CTATCAGGAAGAAATGCAGGAGAT-3', Rv, 5'-GAGCACGCTGAGTACCTCATT-3') were purchased Sigma. For each sample, the real-time PCR reactions were performed in triplicate, and the averages of the obtained C_t values were used for further calculations. Gene expression levels were normalized to the expression of the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was not influenced by the experimental conditions, resulting in the ${}^{\Delta}C_{t}$ value. Gene expression levels were calculated by the comparative C_{t} method ($2^{-\Delta \Delta Ct}$).

4.7. Nitric oxide (NO) assay. The level of nitric oxide was measured in RAW 264.7 cells. 2×10^6 macrophage cells per well were seeded in 6 well plates, incubated 24 hours with or without 10 ng/mL LPS (Sigma, The Netherlands) and 10 ng/mL IFN γ (Sigma-Aldrich, The Netherlands) in the presence or absence of 5 μ M **9c (i472)**. The nitric oxide level in each sample was quantified using commercially available colorimetric nitric oxide assay kit (abcam, ab 65328, UK) following the manufacturer's instructions.

4.8. Lipid peroxidation. RAW 264.7 cells were seeded into a 6-well plate containing 10 $\times 10^6$ cells per well. After overnight incubation, cells were treated with or without 10 ng/mL LPS

(Sigma, The Netherlands) and 10 ng/mL IFN γ (Sigma, The Netherlands) for 24h in the presence or absence of 5 μ M **PD146176**, **Zileuton** or **9c** (**i472**), respectively. Lipid peroxidation was detected by staining with BODIPY 581/591 C11 (Invitrogen, Karlsruhe, Germany) at a final concentration of 2 mM for 30 min at 37 °C. The shift in fluorescence from red to green was analyzed by fluorescence activated cell sorting (FACS) using the Guava Easy Cite 6-2L system (Merck Millipore, Darmstadt, Germany) by excitation at 488 nm. At least three independent experiments were performed per condition.

5. Ancillary information

Supporting information

Includes the docking study with the preferred overlapped poses of compound **9c**, **9f** and **16** (Figure S1), the 2-mercaptoethanol reactivity assay (Figure S2), the LPS toxicity assay (Figure S3), the MTS assay of **9c (i472)** (Figure S4), the FACS of RAW 264.7 cells (Figure S5), and ¹H and ¹³C NMR spectra data (Figure S6).

Molecular formula stings (CSV) are available.

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Notes: The authors declare no competing financial interest.

Abbreviations used

NO, Nitric oxide; 15-LOX-1, 15-lipoxygenase-1; NF-κB, nuclear factor-κB; DAMPs, damage-

associated molecular patterns; TLRs, toll-like receptors; LPS, lipopolysaccharides; iNOS, inducible nitric oxide synthase; ROS, reactive oxygen species; PUFAs, poly-unsaturated fatty acids; AA, arachidonic; LA, linoleic acid; SAR, structure–activity relationships; IFN γ , interferon gamma; EDCI, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; HOBt, *N*-hydroxybenzotriazole (HOBt); IC₅₀ value, 50% inhibition concentration.

Acknowledgements

We acknowledge The Netherlands Organisation for Scientific Research (NWO) for providing VIDI grant (016.122.302 and 723.014.008) to F.J.D. and A.K.H.H., respectively. We would like to thank Bin Liu and Shanshan Song for helpful advice and discussion.

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