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Identification of 2-mercaptohexanoic acids as dual inhibitors of 5-lipoxygenase and microsomal prostaglandin E_2 synthase-1

Christine Greiner [a,†], Heiko Zettl [b,†], Andreas Koeberle [a], Carlo Pergola [c], Hinnak Northoff [d], Manfred Schubert-Zsilavecz [b], Oliver Werz [c,*]

[a] Department of Pharmaceutical Analytics, Pharmaceutical Institute, Eberhard Karls University Tuebingen, Auf der Morgenstelle 8, D-72076 Tuebingen, Germany
[b] Institute of Pharmaceutical Chemistry, ZAFES/OSF, Goethe-University Frankfurt, Max-von-Laue-Str. 9, D-60438 Frankfurt, Germany
[c] Chair of Pharmaceutical/Medicinal Chemistry, Institute of Pharmacy, Friedrich-Schiller-University Jena, Philosophenweg 14, D-07743 Jena, Germany
[d] Institute for Clinical and Experimental Transfusion Medicine, University Medical Center, Tuebingen, Hoppe-Seyler-Straße 3, 72076 Tuebingen, Germany

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ABSTRACT

5-Lipoxygenase (5-LO) and microsomal prostaglandin E_2 synthase (mPGES)-1 are key enzymes in the biosynthesis of leukotrienes and prostaglandin (PG)E_2, respectively, and are considered as valuable targets for the treatment of inflammatory diseases. Here, we present the identification of 2-mercaptohexanoic acid derivatives as dual inhibitors of 5-LO and mPGES-1. The lead compound 2(4-(3-biphenyloxypropoxy)phenylthio)hexanoic acid (**21**) inhibits human 5-LO and mPGES-1 in cell-free assays with an IC_{50} = 3.5 and 2.2 μM, respectively, and suppresses 5-LO in intact cells with even a higher potency (IC_{50} = 0.9 μM). Compound **21** (10 μM) neither significantly inhibited the related 12- or 15-LOs nor cyclooxygenase-1 and -2 or cytosolic phospholipase A_2. Based on the selective and potent inhibition of 5-LO and mPGES-1, further assessment of these 2-mercaptohexanoic acids in preclinical models of inflammation are warranted.

1. Introduction

Prostanoids and leukotrienes (LTs), formed from arachidonic acid (AA) via the cyclooxygenase (COX)-1/2 and 5-lipoxygenase (5-LO) pathway, respectively, are powerful lipid mediators that mediate inflammatory responses, chronic tissue remodelling, cancer, asthma, and autoimmune disorders, but also possess homeostatic functions in the gastrointestinal tract, uterus, brain, kidney, vasculature, and host defence.[1] Whether distinct prostanoids are beneficial or harmful for the body depends on the targeted organ or tissue as well as on the local concentrations of the respective prostanoid. For example, prolonged and excessive formation of prostaglandin (PG)E_2 may cause inflammation, pain, fever and carcinogenesis whereas low amounts of PGE_2 in the stomach or kidney are important for normal physiology of these organs.[2,3] By inhibiting COX-1/2, the non-steroidal anti-inflammatory drugs (NSAIDs) block the synthesis of all prostanoids, including those with protective and beneficial functions (e.g., PGE_2 and PGI_2). Based on the resulting lack of these prostanoids, NSAIDs may cause severe side effects including gastrointestinal injury, renal irritations, and cardiovascular risks.[4] In addition, the concomitant increase of LT formation observed during COX inhibition may contribute to the NSAID-related toxicity.[5,6] Accordingly, anti-inflammatory agents interfering with eicosanoid biosynthesis require a well-balanced pharmacological profile to minimize these on-target side effects. Current anti-inflammatory drug discovery attempts to identify compounds that can suppress the massive formation of pro-inflammatory PGE_2 without affecting homeostatic PGE_2 and PGI_2 synthesis.

To this aim, inhibition of microsomal PGE_2 synthase-1 (mPGES-1) activity[3,7] may provide efficient anti-inflammatory drugs with fewer side effects. In fact, among the three isomeric PGE_2 synthases, the inducible isoform mPGES-1 is essentially the one involved in the overproduction of PGE_2 during inflammation. The concept of selective mPGES-1 inhibition has led to lead compounds of different structural classes, including indole derivatives,[8,9] phenanthrene imidazoles,[10] benzamides,[11] and pirinixic acid derivatives.[12] A novel pharmacological strategy focuses on the dual inhibition of mPGES-1 and 5-LO in order to selectively suppress the synthesis of pro-inflammatory PGE_2 and LTs, respectively.[13–15] Besides some natural products including myrtucommulone,[16] arzanol,[17] garcinol,[18] and curcumin,[19] series of benzo[*g*]indole-3-carboxylates,[9,20] α-*n*-alkyl- or α-aryl-substituted pirinixic acid derivatives,[12] and arylpyrrolizines[14] were shown to block both mPGES-1 and 5-LO, and studies of these compounds in animal models of inflammation revealed high anti-inflammatory efficacy.[17,20,21]

* Corresponding author. Tel.: +49 3641 949801; fax: +49 3641 949802.
E-mail address: oliver.werz@uni-jena.de (O. Werz).
† These authors contributed equally to this work.

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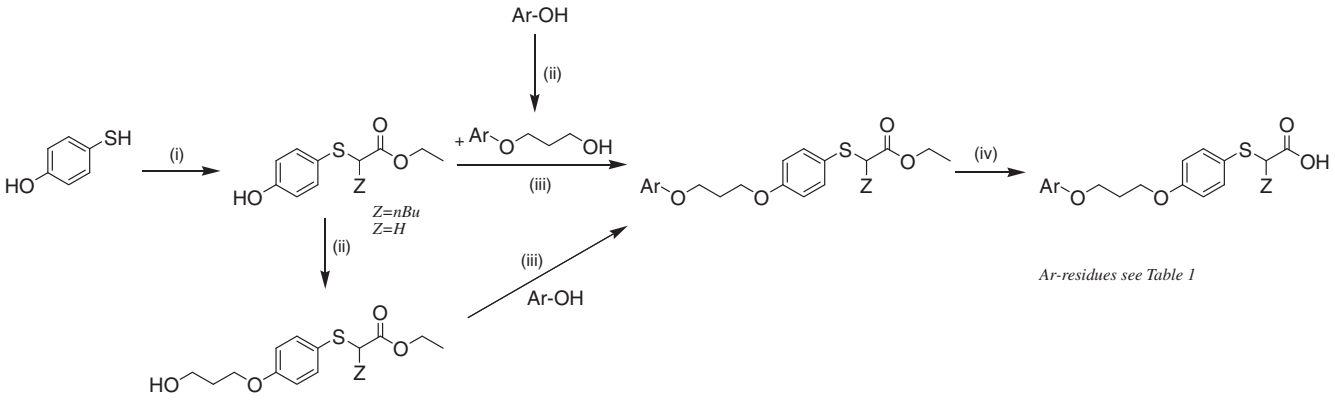
We have recently presented the design, synthesis, and the biological evaluation of novel 2-mercaptohexanoic acid derivatives as activators of peroxisome proliferator-activated receptors (PPARs) α and γ.[22] Here we present the identification of these 2-mercaptohexanoic acid derivatives as dual inhibitors of 5-LO and mPGES-1 that efficiently suppress the formation of LTs and PGE_2 without marked inhibition of the cellular synthesis of other oxygenated metabolites generated by related enzymes including COX, 12/15-LO, and $cPLA_2$. In contrast to mPGES-1 inhibitors that have just begun to be developed, agents that suppress 5-LO product synthesis have been the subject of intensive research efforts over the past 30 years and their molecular pharmacology is well characterized. Thus, the efficient inhibition of 5-LO by the lead compound **21** in a cell-free and a cell-based assay prompted for more detailed analysis of the interference with 5-LO and revealed an advantageous pharmacological profile of this compound.



Scheme 1. Synthesis of 2-mercaptohexanoic acid derivatives and of the 2-mercaptoacetic acid derivative (**1**). Reagents and conditions: (i) ethyl-2-bromoacetate ($Z = H$) or ethyl-2-bromohexanoate ($Z = nBu$), Et_3N, $CHCl_3$, reflux, 1 h; (ii) 3-bromopropan-1-ol, K_2CO_3, ACN, reflux, 24–48 h; (iii) DEAD or ADDP, TPP, THF, rt, 4–48 h; (iv) LiOH, MeOH, H_2O, 40 °C, 2 h.

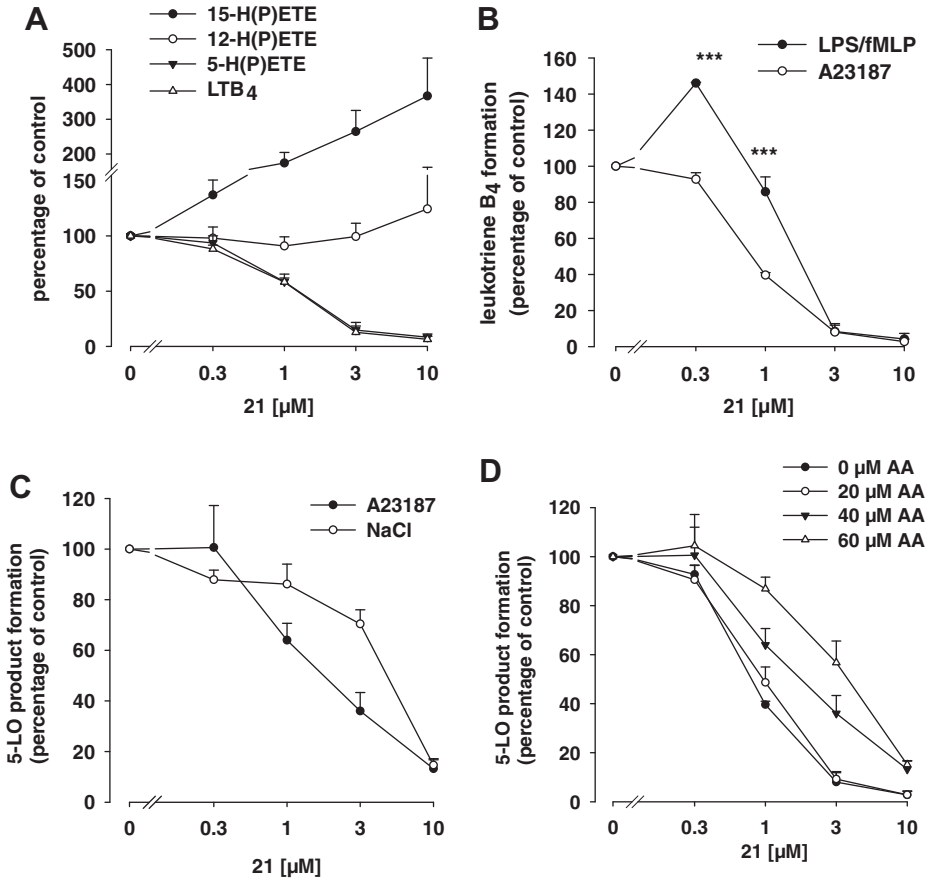
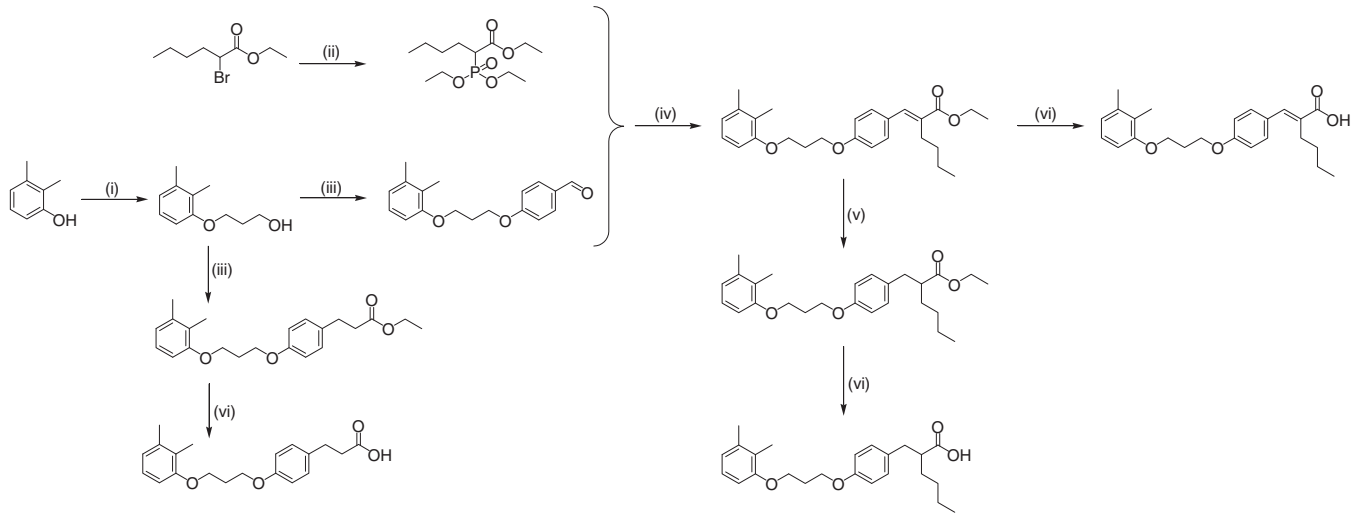


Figure 1. Inhibition of 5-LO activity by compound **21** in cell-based assays. (A) Inhibition of 5-LO, 12-LO and 15-LO in human PMNL stimulated with 2.5 μM A23187 plus 20 μM AA. (B) Comparison of 5-LO inhibition by **21** between PMNL challenged with 2.5 μM A23187 or with 1 μg/ml LPS and 100 nM fMLP. (C) Comparison of 5-LO inhibition by **21** between PMNL challenged with 2.5 μM A23187 or 0.3 M NaCl in the presence of 40 μM AA, each. (D) Inhibition of 5-LO in PMNL stimulated with 2.5 μM A23187 together with exogenous AA at varying concentrations. Data are mean ± SE, $n = 3$-4. $^{***}p < 0.001$ for samples treated with A23187 versus those treated with LPS/fMLP.



Scheme 2. Synthesis of carbon analogs **5**, **6** and **7**. Reagents and conditions: (i) 3-bromopropan-1-ol, K_2CO_3, ACN, reflux, 24 h; (ii) triethylphosphite, reflux, overnight; (iii) 4-hydroxybenzaldehyde or ethyl 3-(4-hydroxyphenyl)propanoate, DEAD, TPP, THF, rt, 4 h; (iv) NaH, THF, rt, 24 h; (v) Pd/C, H_2, EtOH, rt, 24 h; (vi) LiOH, MeOH, H_2O, reflux, 8–20 h.

2. Results and discussion

2.1. Chemistry

The synthesis of the presented compounds **1**–**24** has been described previously.[22] The 2-mercaptohexanoic acid derivatives were synthesized starting with 4-mercaptophenol as shown in Scheme 1. First, nucleophilic substitution with α-bromoesters yielded the thioether derivatives. The lipophilic backbone of the compounds is arranged by two ether syntheses (by coupling with 3-bromopropan-1-ol and by a Mitsunobu reaction with commercially available phenols). Finally, hydrolysis of the ester group yielded the desired carboxylic acids. Enantioselective resolution of **2** was achieved by enantioselective preparative HPLC as described.[23] The carbon analogs (**6** and **7**) were prepared starting with the conversion of ethyl 2-bromohexanoate into its phosphonate derivative by an Arbuzov reaction (Scheme 2). The product was coupled by a Wittig–Horner reaction to the aldehyde group of a precursor derived from 4-hydroxybenzaldehyde. The resulting ester was directly hydrolyzed (to yield **7)** as well as hydrogenated and afterwards hydrolyzed (to yield **6**). Only two steps (Mitsunobu condensation and hydrolysis) were needed for the preparation of the α-unsubstituted carbon analog **5**.

2.2. Biology

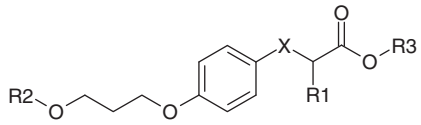
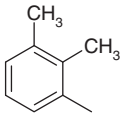
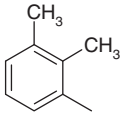
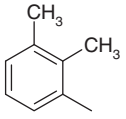
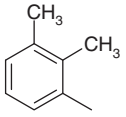
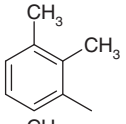
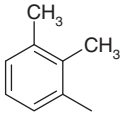
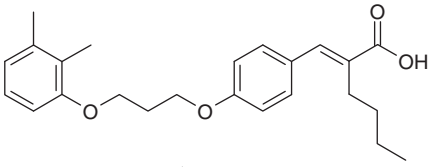
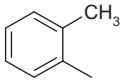
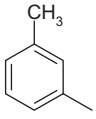
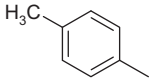
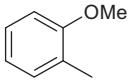
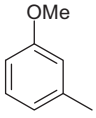
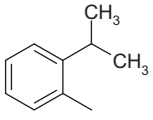
2.2.1. Screening of dual inhibitors of 5-LO and mPGES-1

First, the test compounds were assayed for their ability to inhibit mPGES-1 and 5-LO in cell-free test systems. For analysis of mPGES-1 inhibition, microsomal preparations of IL-1β-stimulated A549 cells (5 μg/100 μl) were pre-incubated with the test compounds and assayed for suppression of enzymatic PGE_2 formation from PGH_2 at standardized assay conditions (i.e., potassium phosphate buffer 0.1 M, pH 7.4, 20 μM PGH_2 as substrate, and 2.5 mM glutathione as co-substrate[24]), modified according to Jakobsson et al.[25] Since the cyclic peroxide PGH_2 is highly reactive and spontaneously degrades non-enzymatically to PGF_2, PGD_2, and PGE_2 in aqueous solutions within a few minutes,[26] the enzymatic assay was performed at 4 °C for 1 min in order to avoid non-enzymatic conversion of PGH_2 to PGE_2. For analysis of 5-LO inhibition, human recombinant 5-LO enzyme was expressed in *Escherichia coli* and purified by affinity chromatography, and 5-LO activity was assayed using 20 μM AA as substrate.[27] The indole derivative **26** (MK886), a well-recognized inhibitor of mPGES-1,[28] and the selective iron ligand-type 5-LO inhibitor **25** (BWA4C[29]) were used as controls.

The test compounds were first analysed at a concentration of 10 μM. IC_{50} values were determined for those compounds that suppressed enzyme activity by more than 50%, whereas the remaining enzyme activity is given for the others (Table 1). Test concentrations >10 μM in cell-based assays were not considered because of inconsistent solubility behavior in the aqueous assay buffers at higher concentrations, and thus assessment of IC_{50} values of poor inhibitors (IC_{50} >10 μM) was not further explored. As shown in Table 1, the 2-substituted acetic acid derivative **1** caused only weak inhibition of both mPGES-1 and of 5-LO, whereas elongation of the alkyl chain to the corresponding hexanoic acid **2** yielded an efficient dual 5-LO/mPGES-1 inhibitor. Of interest, there was no significant difference in the potencies of the *(R)*-(**3**) and the *(S)*-(**4**) enantiomers implying that the absolute configuration of the α-C atom is negligible. A loss of effectiveness was observed after replacing the sulfur of **1** or of **2** by carbon yielding **5** and **6**, as well as in the corresponding alkene derivative of **6** (α-(*n*)-butyl-substituted cinnamonic acid derivative **7**). Interestingly, removal of the two methyl moieties from the phenyl ring of **2** (yielding **8**) was clearly detrimental. Single methylation partially improved the inhibition for mPGES-1 regardless of the positioning (in *ortho* (**9**), *meta* (**10**), *para* (**11**)) in the phenyl moiety, though the compounds were less potent than the double (2,3-) methylated **2**. Also insertion of oxygen yielding the 2-methoxy- (**12**) and the 3-methoxy- (**13**) derivatives of **8** was essentially without effect. On the other hand, enlargement of the alkyl substituent such as introduction of a 2-isopropyl (**14**) or 3-isopropyl (**15**) residue significantly increased the efficiency over the corresponding methyl-substituted analogs **9** and **10**, respectively. Also replacement of the 3-methyl group in **10** by a trifluoromethyl residue (**16**) enhanced both inhibition of 5-LO and of mPGES-1, and the potency to inhibit 5-LO could be further improved by introducing chlorine in 4-position (**17**). Interestingly, the 4-chloro-2-methoxy-phenyl derivative **18** was significantly less efficient. On the other hand, the bicyclic derivatives **19** and **21** were active, whereas upon replacement by a quinoline moiety (**23**), the inhibitory effect was lost. Taken together, **17** was the most efficient inhibitor for both mPGES-1 and 5-LO with IC_{50} values of 1.7 and 2 μM, respectively.

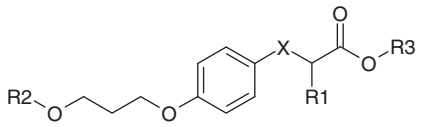
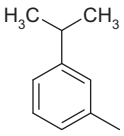
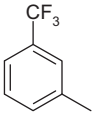
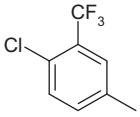
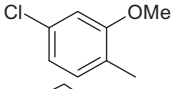
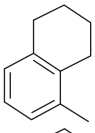
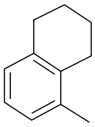
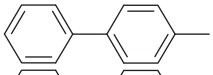
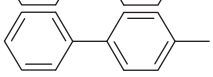
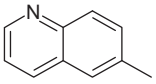
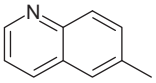
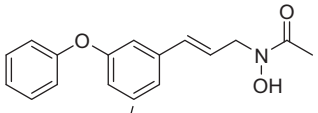
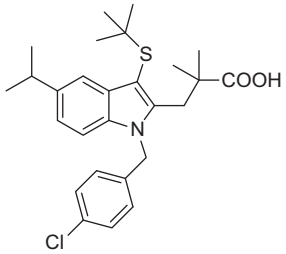
In order to analyze whether the compounds penetrate through the cell membrane and are active also in the cellular context, interference with 5-LO product formation was assessed in human

Table 1
Inhibition of mPGES-1 and 5-LO in cell-free assays by test compounds and suppression of 5-LO product synthesis in intact cells (expressed as IC_{50} values, mean of $n = 3–5$ experiments)

Compd					mPGES-1 IC_{50} in [μM] (remaining activity at 10 μM ± SE [%])	5-LO cell-based IC_{50} in [μM] (remaining activity at 10 μM ± SE [%])	5-LO cell-free IC_{50} in [μM] (remaining activity at 10 μM ± SE [%])
	X	**R1**	**R2**	**R3**			
1	*S*	H		H	>10 (75.4 ± 0.9)	>10 (69.6 ± 9.8)	>10 (72.4 ± 4.0)
2	*S*	Butyl		H	2.9	3.0	8.8
3	*S*	(*R*)-Butyl		H	4.2	1.1	10.0
4	*S*	(*S*)-Butyl		H	4.6	2.0	8.2
5	CH_2	H		H	>10 (83.9 ± 14.2)	>10 (80.0 ± 2.0)	>10 (81.8 ± 4.4)
6	CH_2	Butyl		H	7.1	2.0	>10 (70.8 ± 5.0)
7					>10 (28.3 ± 10.3)	1.6	>10 (88.9 ± 2.7)
8	*S*	Butyl		H	>10 (81.9 ± 5.6)	9.5	>10 (69.4 ± 4.8)
9	*S*	Butyl		H	8.8	4.8	>10 (58.3 ± 6.8)
10	*S*	Butyl		H	>10 (58.0 ± 5.5)	4.8	>10 (79.3 ± 10.6)
11	*S*	Butyl		H	9.3	5.2	>10 (67.6 ± 11.7)
12	*S*	Butyl		H	>10 (88.3 ± 3.0)	>10 (57.4 ± 11.9)	>10 (79.4 ± 6.4)
13	*S*	Butyl		H	>10 (59.1 ± 6.5)	4.8	>10 (67.7 ± 5.8)
14	*S*	Butyl		H	4.1	1.6	7.8

(*continued on next page*)

Table 1 (*continued*)

Compd					mPGES-1 IC_{50} in [μM] (remaining activity at 10 μM ± SE [%])	5-LO cell-based IC_{50} in [μM] (remaining activity at 10 μM ± SE [%])	5-LO cell-free IC_{50} in [μM] (remaining activity at 10 μM ± SE [%])
	X	**R1**	**R2**	**R3**			
15	*S*	Butyl		H	2.7	1.4	6.0
16	*S*	Butyl		H	2.0	1.6	9.8
17	*S*	Butyl		H	1.7	1.5	2.0
18	*S*	Butyl		H	9.4	>10 (79.3 ± 14.0)	>10 (59.2 ± 7.4)
19	*S*	Butyl		H	2.2	1.4	4.5
20	*S*	Butyl		C_2H_5	nd	>10 (87.4 ± 2.8)	>10 (79.4 ± 28.4)
21	*S*	Butyl		H	2.2	0.9	3.5
22	*S*	Butyl		C_2H_5	nd	>10 (91.5 ± 0.9)	>10 (83.0 ± 38.7)
23	*S*	Butyl		H	>10 (ni)	>10 (92.2 ± 3.4)	>10 (59.0 ± 12.7)
24	*S*	Butyl		C_2H_5	>10 (83.9 ± 3.6)	0.8	>10 (68.3 ± 12.8)
25	BWA4C				nd	0.08	0.16
26	MK886				2.4	0.09	ni at 1 μM

For compounds with IC_{50} >10 μM, the remaining enzymatic activity (in %) is given at 10 μM.
ni = no inhibition.
nd = not done.

polymorphonuclear leukocytes (PMNL) stimulated with A23187 plus exogenous AA (20 μM) which is a suitable and convenient cell-based model to study 5-LO inhibitors.[30] The 5-LO products (i.e., LTB_4 and its trans-isomers and 5(*S*)-hydro(pero)xy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-H(P)ETE)) were extracted and analysed by RP-HPLC as described.[31] For mPGES-1 inhibitors, a direct test system that allows select analysis of interference with endogenous mPGES-1 activity in the cell (i.e., the distinct transformation of PGH_2 to PGE_2) is not available. We observed that for inhibition of 5-LO product synthesis in intact cells, the compounds

were consistently more efficient than in cell-free assays, with the exception of the quinoline-substituted compound **23** that essentially failed in intact cells. For example, the IC_{50} values of **16** or **21** were 9.8 and 3.5 μM for isolated 5-LO but 1.6 and 0.9 μM in intact PMNL, respectively. Intriguingly, esterification of **21** and **19** leading to **22** and **20**, respectively, caused a strong loss of efficiency in intact cells as well as in cell-free assays. On the other hand, the corresponding ethyl ester of the inactive **23**, that is, compound **24** turned out to be a potent 5-LO inhibitor in intact cells (but not in cell-free assays) with an IC_{50} = 0.8 μM, which is not readily understood. Together, 2-mercaptohexanoic acids with large and lipophilic aryloxy residues exemplified by the leads **17**, **19** and **21** represent potent dual inhibitors of 5-LO and mPGES-1.

In view of their structure, the active 2-mercaptohexanoic acids may resemble long chain fatty acids such as AA. Since 5-LO binds AA as substrate in the active site and AA is an inhibitor of mPGES-1 (IC_{50} = 0.3 μM), it appeared reasonable that these 2-mercaptohexanoic acids act as AA mimetics and bind into the active sites of mPGES-1 and of 5-LO. In view of such considerations, besides 5-LO and mPGES-1, other enzymes within the AA cascade could be affected by the 2-mercaptohexanoic acids. Hence, we investigated the effects of the lead compounds **17**, **19** and **21** on the activities of isolated COX-1 and -2, and (at least for **21**) on isolated $cPLA_2$. Reference inhibitors were indomethacin for COX-1, celecoxib for COX-2 and the $cPLA_{2\alpha}$ inhibitor *N*-{(2*S*,4*R*)-4-(biphenyl-2-ylmethyl-isobutyl-amino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl}-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl]acrylamide that blocked the respective enzyme activities as expected (not shown). The results presented in Table 2 show that none of the 2-mercaptohexanoic acid derivatives possess a significant inhibitory effect on any of these enzymes, suggesting selectivity for 5-LO and mPGES-1.

2.2.2. Analysis of the mechanism of 5-LO inhibition by compound 21

Based on their molecular mode of action, 5-LO inhibitors can be categorized in general as redox-type inhibitors, iron ligand-type inhibitors, non-redox-type 5-LO inhibitors and agents with distinct modes of 5-LO inhibition.[30] The lack of moieties with reducing properties in the structures of **17**, **19** or **21** rather excludes a redox- or radical scavenging-based 5-LO inhibitory mechanism, and also typical iron-chelating moieties of prototype iron-ligand inhibitors are absent. In order to gain more insights into the molecular mode of action, we performed mechanistic studies with compound **21,** the most attractive 5-LO inhibitor in cell-based (IC_{50} = 0.9 μM) and cell-free assays (IC_{50} = 3.5 μM) from our SAR investigations.

Table 2
Inhibition of COX-1 and COX-2 and of cPLA2 by the compounds **17**, **19** and **21** at a concentration of 10 μM, *n* = 3

Compd	COX-1 (remaining activity at 10 μM ± SE [%])	COX-2 (remaining activity at 10 μM ± SE [%])	$cPLA_2$ (remaining activity at 10 μM ± SE [%])
17	71.3 ± 2.0	ni	nd
19	ni	ni	nd
21	79.2 ± 8.8	ni	105.5 ± 19.0

ni = no inhibition.
nd = not done.

Preparations of PMNL, isolated from human peripheral blood, generate not only 5-LO products but also transform AA via platelet-type 12-LO (present in PMNL adherent platelets) and 15-LO (in eosinophilic granulocytes) to 12(*S*)-hydro(pero)xy-5,8-cis-10-trans-14-cis-eicosatetraenoic acid (12(*S*)-H(P)ETE) and 15(*S*)-hydro(pero)xy-5,8,11-cis-13-trans-eicosatetraenoic acid (15(*S*)-H(P)ETE). As shown from Figure 1A, compound **21** caused no significant suppression of 12(*S*)-H(P)ETE and 15(*S*)-H(P)ETE in PMNL up to 10 μM, again supporting selectivity for 5-LO/mPGES-1.

The structural properties led us to hypothesize that **21** may act as non-redox-type 5-LO inhibitor. Because the potency of non-redox-type 5-LO inhibitors can vary depending on the stimulus and signalling pathway inducing 5-LO product synthesis,[32] we analysed the potency of **21** on 5-LO product formation in PMNL stimulated with A23187 and in cells challenged with fMLP upon priming with LPS. Whereas A23187 activates 5-LO via a Ca^{2+}-dependent mechanism, LPS/fMLP may partially act by Ca^{2+} but also via induction of 5-LO phosphorylation by mitogen-activated protein kinases (MAPK).[33] In fact, non-redox-type 5-LO inhibitors were shown to be less potent when 5-LO is activated by phosphorylation.[32] As shown in Figure 1B, the potency of **21** was somewhat decreased upon stimulation with LPS/fMLP as compared to A23187. Moreover, we studied the efficiency of **21** in PMNL exposed to 300 mM NaCl that causes cell stress leading to 5-LO activation by phosphorylation independent of Ca^{2+}. However, no striking differences in the potency of **21** were evident (Fig. 1C). Next, we tested whether the substrate concentration may influence the 5-LO inhibitory effect of **21**, again a phenomenon expected for competitive, non-redox-type 5-LO inhibitors.[34] The potency of **21** is only slightly reduced when exogenous AA was provided in excess (up to 40), suggesting that **21** inhibits 5-LO essentially independent of the substrate concentration (Fig. 1D).

Besides cell-based analysis, studies with **21** were performed in cell-free assays. Wash-out experiments (dilution from 10 to 1 μM) demonstrated that the inhibitory effect of **21** on isolated 5-LO can be (at least partially) reverted (Fig. 2A), implying that the compound does not irreversibly inactivate 5-LO or acts by covalent binding to it. The potency of non-redox-type 5-LO inhibitors is typically impaired at elevated hydroperoxide levels.[35,36]

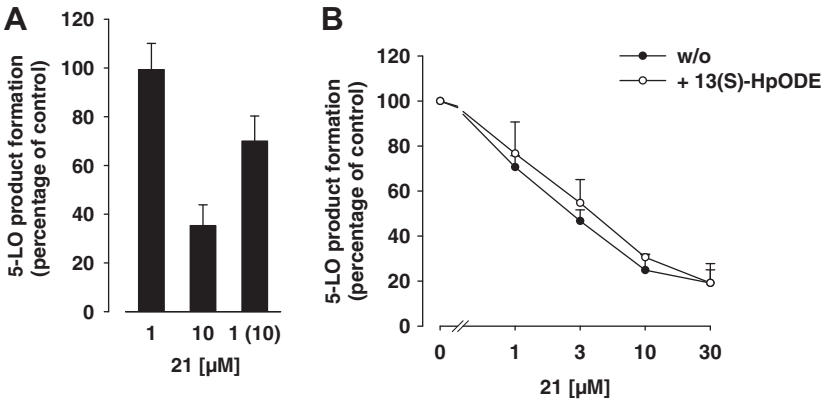


Figure 2. Inhibition of 5-LO activity by compound **21** in cell-free assays. (A) Inhibition of isolated human recombinant 5-LO by **21** is partially reversible upon 10-fold dilution. (B) Impact of 13(*S*)-HpODE on the potency of **21** to inhibit isolated 5-LO. Data are mean ± SE, *n* = 4–5.

Hence, we analyzed inhibition of 5-LO by **21** in the absence and presence of 1 μM 13(*S*)-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13(*S*)-HpODE). As can be seen from Figure 2B, the potency of **21** was not affected by 13(*S*)-HpODE. Thus, **21** does not exhibit typical properties of non-redox-type 5-LO inhibitors such as ZM230487 or CJ-13610.[35,36]

2.3. Discussion

One current pharmacological strategy aiming to overcome NSAID-related toxicity pursues the dual interference with mPGES-1 and 5-LO. In contrast to COX inhibition, interference with mPGES-1 is thought to selectively suppress the pathological PGE_2 generation thereby maintaining the house-keeping functions of other prostanoids.[13] Moreover, experimental evidence from investigations of NSAIDs in vitro and in vivo suggests that the elevated LT formation due to target-related inhibition of prostanoid biosynthesis contributes to the toxicity of NSAIDs.[5,6] Here, we identified simple 2-mercaptohexanoic acid derivatives as dual mPGES-1/5-LO inhibitors with IC_{50} values in the low micromolar or even submicromolar range. The lead compounds (i.e., **17, 19** and **21**), elongated with lipophilic bulky aryloxy moieties, have a well-balanced activity on both enzymes and, at least concerning 5-LO, are active also in intact cells with even improved efficiency. Elucidation of the molecular mode of action of 5-LO inhibition revealed no congruent mechanism with other typical 5-LO inhibitors such as redox-active agents, iron-ligands or non-redox-type/competitive 5-LO inhibitors. Importantly, the lead **21** showed advantageous features in terms of 5-LO inhibition such as no marked loss of potency at elevated peroxide tone and at increased substrate concentrations, and no strong dependency on the stimulus. Moreover, **21** does not inhibit COX-1 and -2, $cPLA_2$ and other human LOs (i.e., 12- and 15-LO). Only few synthetic dual 5-LO/mPGES-1 inhibitors have been described thus far[13,15] and data related to gastrointestinal, renal or cardiovascular toxicity of these substances in animal models are still missing. At least, two recent studies of dual mPGES-1/5-LO inhibitors (indole-3-carboxylates[9] and pirinixic acid derivatives[37] in vivo provided encouraging results in terms of anti-inflammatory efficacy. Together, the novel lead structures presented in this study may serve as potential candidates for intervention with inflammatory diseases and deserve further exploration in preclinical studies.

3. Experimental

3.1. Compounds and chemistry

Compounds **1–24** were synthesized as previously reported.[22] All structures were confirmed by ^1H and ^{13}C NMR, as well as by mass spectrometry (ESI). The purity (>98%) was checked by combustion analysis as described.[22]

3.2. Assay systems

3.2.1. Materials

The $cPLA_{2\alpha}$ inhibitor *N*-{(2*S*,4*R*)-4-(biphenyl-2-ylmethyl-isobutyl-amino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-yl methyl}-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl] acrylamide was from Calbiochem (Bad Soden, Germany). DMEM/High Glucose (4.5 g/l) medium, penicillin, streptomycin, trypsin/EDTA solution, PGH_2, Larodan (Malmö, Sweden); 11β-PGE_2, PGB_1, MK886, [5,6,8,9,11,12,14,15-^{3}H] arachidonic acid ([^{3}H]AA), BioTrend Chemicals GmbH (Cologne, Germany); Ultima Gold™ XR, Perkin Elmer (Boston, MA). All other chemicals were obtained from Sigma–Aldrich (Deisenhofen, Germany) unless stated otherwise.

3.2.2. Cells and cell viability assay

Human PMNL were freshly isolated from leukocyte concentrates obtained at the Blood Center of the University Hospital Tuebingen (Germany). In brief, venous blood was taken from healthy adult donors and leukocyte concentrates were prepared by centrifugation at 4000×*g* for 20 min at 20 °C. PMNL were immediately isolated by dextran sedimentation, centrifugation on Nycoprep cushions (PAA Laboratories, Linz, Austria), and hypotonic lysis of erythrocytes as described previously.[38] Cells were finally resuspended in phosphate-buffered saline pH 7.4 (PBS) containing 1 mg/ml glucose and 1 mM $CaCl_2$ (PGC buffer).

A549 cells were cultured in DMEM/High glucose (4.5 g/l) medium supplemented with heat-inactivated fetal calf serum (10%, v/v), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37 °C in a 5% CO_2 incubator. After 3 days, confluent cells were detached using 1 × trypsin/EDTA solution and reseeded at 2×10^6 cells in 20 ml medium in 175 cm^2 flasks. Cell viability was measured using the colorimetric MTT dye reduction assay. A549 cells (4×10^4 cells/100 μl medium) were plated into a 96-well microplate and incubated at 37 °C and 5% CO_2 for 16 h. Then, test compounds (10 μM, each) or solvent (DMSO, never exceeding a final concentration of 0.3%) were added, and the samples were incubated for another 5 h. MTT (20 μl, 5 mg/ml) was added and the incubations were continued for 4 h. The formazan product was solubilized with SDS (10%, m/v in 20 mM HCl) and the absorbance of each sample was measured at 595 nm relative to the absorbance of vehicle (DMSO)-treated control cells using a multiwell scanning spectrophotometer (Victor^3 plate reader, PerkinElmer, Rodgau-Juegesheim, Germany). None of the compounds significantly reduced cell viability at a concentration of 10 μM (data not shown), excluding possible acute cytotoxic effects of the compounds in the cellular assays.

3.2.3. Determination of product formation by 5-LO, 15-LO and 12-LO in cell-based assays

For assays of intact cells stimulated with ionophore A23187 or NaCl, 5×10^6 freshly isolated PMNL were resuspended in 1 ml PGC buffer. After preincubation with the compounds for 15 min at 37 °C, 5-LO product formation was started by addition of 2.5 μM A23187 plus exogenous AA (at the indicated concentrations) or 0.3 M NaCl was supplemented 3 min before addition of AA. After 10 min at 37 °C, the reaction was stopped with 1 ml of methanol and 30 μl of 1 N HCl, 200 ng prostaglandin B_1 and 500 μl of PBS were added. Formed 5-LO metabolites were extracted and analyzed by HPLC as described.[39] 5-LO product formation is expressed as ng of 5-LO products per 10^6 cells, which includes LTB_4 and its all-trans isomers, 5(*S*),12(*S*)-di-hydroxy-6,10-trans-8,14-cis-eicosatetraenoic acid (5(*S*),12(*S*)-DiHETE), and 5-H(P)ETE. Cysteinyl $LTsC_4$, D_4 and E_4 were not detected, and oxidation products of LTB_4 were not determined. 15-H(P)ETE was analyzed as product of 15-LO and 12-H(P)ETE as product of 12-LO. To assess 5-LO product formation in cells stimulated with fMLP, 2×10^7 PMNL were resuspended in 1 ml PBS/glucose, primed with 1 μg/ml LPS for 10 min at 37 °C and 0.3 U/ml adenosine deaminase was added. After another 10 min, cells were treated with the compounds for 10 min and 1 μM fMLP was added. The reaction was stopped on ice after 5 min and cells were centrifuged (800×*g*, 10 min). Formed LTB_4 was determined by ELISA according to the manufacturer's protocol (Assay Designs, Ann Arbor, MI).

3.2.4. Expression and purification of human recombinant 5-LO from *E. coli*, and determination of 5-LO activity in cell-free systems

E. coli BL21 was transformed with pT3-5LO plasmid, and recombinant 5-LO protein was expressed at 37 °C, and 5-LO was purified

as described.[32] Purified 5-LO was immediately used for 5-LO activity assays. Aliquots of purified 5-LO (0.5 μg) were added to 1 ml PBS/EDTA plus 1 mM ATP and pre-incubated with the test compounds. After 5–10 min at 4 °C, samples were pre-warmed for 30 s at 37 °C, and 2 mM $CaCl_2$, and the indicated amounts of AA (routinely 20 μM AA) with or without 1 μM 13(*S*)-HpODE were added to start 5-LO product formation. The reaction was stopped after 10 min at 37 °C by addition of 1 ml ice-cold methanol and the formed metabolites were analyzed by HPLC as described for intact cells.

3.2.5. Induction of mPGES-1 expression in A549 cells, isolation of microsomes, and determination of PGE_2 synthase activity

Preparation of A549 cells and determination of mPGES-1 activity was performed as described previously.[24] In brief, A549 cells (2×10^6 cells in 20 ml medium) were plated in 175 cm^2 flasks and incubated for 16 h at 37 °C and 5% CO_2. Subsequently, the culture medium was replaced by fresh DMEM/High glucose (4.5 g/l) medium containing fetal calf serum (2%, v/v). In order to induce mPGES-1 expression, IL-1β (1 ng/ml) was added, and cells were incubated for another 72 h. Thereafter, cells were detached with trypsin/EDTA, washed with PBS, and frozen in liquid nitrogen. Ice-cold homogenisation buffer (0.1 M potassium phosphate buffer pH 7.4, 1 mM phenylmethanesulfonylfluoride, 60 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin, 2.5 mM glutathione, and 250 mM sucrose) was added, and after 15 min, cells were resuspended and sonicated on ice (3×20 s). The homogenate was subjected to differential centrifugation at 10,000×*g* for 10 min and 174,000×*g* for 1 h at 4 °C. The pellet (microsomal fraction) was resuspended in 1 ml homogenization buffer, and the total protein concentration was determined by Coomassie protein assay. Microsomal membrane fractions were stored at −80 °C for several weeks. Microsomal membranes were diluted in potassium phosphate buffer (0.1 M, pH 7.4) containing 2.5 mM glutathione to give a final concentration of 50 μg/ml. Test compounds or vehicle (DMSO at a final concentration of 1%) were added, and after 15 min at 4 °C, the reaction (100 μl total volume) was initiated by addition of PGH_2 (20 μM, final concentration). After 1 min at 4 °C, the reaction was terminated using stop solution (100 μl; 40 mM $FeCl_2$, 80 mM citric acid, and 10 μM of 11β-PGE_2). PGE_2 was separated by solid phase extraction on reversed phase (RP)-C18 material as previously described.[24] 11β-PGE_2 was used as internal standard to quantify PGE_2 product formation by integration of the area under the peaks.

3.2.6. Activity assays of isolated COX-1 and -2

Inhibition of the activities of isolated COX-1 and COX-2 was performed as described.[24] Briefly, purified COX-1 (ovine, 50 units) or COX-2 (human recombinant, 20 units) were diluted in 1 ml reaction mixture containing 100 mM Tris buffer pH 8, 5 mM glutathione, 5 μM haemoglobin, and 100 μM EDTA at 4 °C and pre-incubated with the test compounds for 5 min. Samples were pre-warmed for 60 s at 37 °C, and AA (5 μM for COX-1, 2 μM for COX-2) was added to start the reaction. After 5 min at 37 °C, 12(*S*)-hydroxy-5*Z*,8*E*,10*E*-heptadecatrienoic acid was extracted and then analyzed by HPLC.

3.2.7. Statistics

Data are expressed as mean ± SE. IC_{50} values were graphically calculated from averaged measurements at five different concentrations of the compounds (routinely 0.1–10 μM) using SigmaPlot 9.0 (Systat Software Inc., San Jose, USA). The program Graphpad Instat (Graphpad Software Inc., San Diego, CA) was used for statistical comparisons. Statistical evaluation of the data was performed by one-way ANOVAs for independent or correlated samples followed by Tukey HSD post-hoc tests. Where appropriate, Student's *t* test for paired and correlated samples was applied. A p value of <0.05 (∗) was considered significant.

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