Brief Articles

Novel and Potent Inhibitors of 5-Lipoxygenase Product Synthesis Based on the Structure of Pirinixic Acid

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A novel class of potent 5-lipoxygenase (5-LO) product synthesis inhibitors based on the structure of pirinixic acid (4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid, compound **1**) is presented. Systematic profiling of **1**, i.e., esterification of the carboxylic acid, α-substitution, and replacement of the *o*-dimethylaniline by 6-aminoquinoline, leads to potent suppressors of 5-LO product formation in activated polymorphonuclear leukocytes, exemplified by ethyl 2-[4-chloro-6-(quinoline-6-ylamino)-pyrimidin-2-ylsulfanyl]octane-1-carboxylate (**6d**, $IC_{50} = 0.6\ \mu M$). These derivatives may possess potential for intervention with inflammatory and allergic diseases.

Introduction

Metabolites derived from the 5-lipoxygenase (5-LO[a]) pathway, in particular the leukotrienes (LTs), exert their biological effects via specific G protein-coupled receptors and play pivotal roles in inflammatory and allergic disorders as well as in cardiovascular disease (CVD) and cancer.[1,2] 5-LO, a nonheme iron-containing dioxygenase, catalyzes the first steps in the conversion of arachidonic acid (AA) into LTA_4 under the aid of the nuclear membrane-bound 5-LO-activating protein (FLAP).[3] LTA_4 is further converted by LTA_4 hydrolase into LTB_4, which is a potent chemotactic agent and activator for phagocytes. Alternatively, conjugation of LTA_4 with reduced glutathione by LTC_4 synthases yields the cysteinyl-containing LTs C_4, D_4, and E_4, which cause bronchoconstriction and vascular permeability.[1] According to its role as key enzyme in the biosynthesis of all LTs, 5-LO represents a valuable target for pharmacological intervention with diseases such as asthma and allergic rhinitis, CVD, as well as certain types of cancer.[2] Today, LT antagonists are successfully used in asthma therapy, whereas the development of LT synthesis inhibitors is less advanced.[4]

Pirinixic acid (4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid, compound **1**) belongs to the fibrates acting as agonist of the peroxisome proliferator-activated receptor (PPAR)[a], a ligand-dependent nuclear transcription factor that essentially reduces inflammatory responses.[5,6] Thus, anti-inflammatory effects of **1** have been reported in numerous cellular and animal models of inflammation and CVD that rely on slow-acting transcriptional events.[7−9] In contrast to the well-established anti-inflammatory actions of **1** mediated by genomic pathways modulating protein expression, little is known about short-term effects of **1** on cellular functions related to inflammation.

Recently, we presented that aliphatic α-substitution of **1** enhances both PPARα and PPARγ agonism.[10,11] Here, we address the effectiveness of **1** and its derivatives for inhibition of 5-LO product synthesis and interaction with 5-LO. Although **1** itself failed to suppress 5-LO activity, we demonstrate that target-oriented structural modifications of **1** lead to potent inhibitors of LT biosynthesis that efficiently interfere with 5-LO activity in intact cells and to a minor extent also in cell-free systems.

Results and Discussion

For LT biosynthesis, AA is released by $cPLA_2$ and converted by activated 5-LO aided by FLAP.[3] Activation of 5-LO in the cell requires Ca^{2+} mobilization and/or 5-LO phosphorylation by protein kinases that cause translocation of 5-LO from soluble locales to the nuclear membrane close to FLAP.[3] For conclusive analysis of the effectiveness of the test compounds, both a cell-based test system using isolated human polymorphonuclear leukocytes (PMNL) and a cell-free assay utilizing crude human recombinant 5-LO expressed in *Escherichia coli* were applied. In the cell-based assays, many possibilities aside from direct interference with 5-LO exist (e.g., FLAP or $cPLA_2$ inhibition, suppression of 5-LO kinases or Ca^{2+} mobilization, inhibition of 5-LO translocation), eventually suppressing LT synthesis.[12]

To evaluate the compounds in intact PMNL, the direct 5-LO products 5(*S*)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid and the trans- and epi-trans isomers of LTB_4, as well as LTB_4, were analyzed. Cells were preincubated with the compounds at 1 and 10 μM, and subsequently 5-LO product formation was elicited by 2.5 μM Ca^{2+} ionophore A23187 (5-methylamino-2-(2*S*,3*R*,5*R*,8*S*,9*S*)-3,5,9-trimethyl-2-(1-oxo-1-(1*H*-pyrrol-2-yl)propan-2-yl)-1,7-dioxaspiro(5.5)undecan-8-yl)methyl)benzooxazole-4-carboxylic acid). Exogenous AA (20

* To whom correspondence should be addressed. Phone: ++49-7071-2978793. Fax: ++49-7071-294565. E-mail: oliver.werz@uni-tuebingen.de.
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[a] Abbreviations: AA, arachidonic acid; CVD, cardiovascular disease; FLAP, 5-LO-activating protein; 5-LO, 5-lipoxygenase; LT, leukotriene; PPAR, peroxisome proliferator-activated receptor; PA, pirinixic acid; PMNL, polymorphonuclear leukocytes.

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Table 1. Inhibition of 5-LO Product Formation by Test Compounds in Intact PMNL[c]

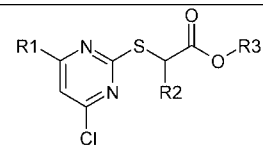
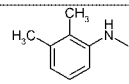
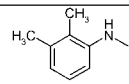
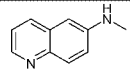
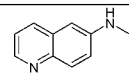
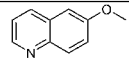
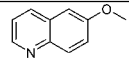
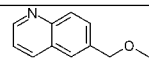
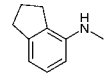
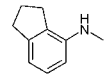
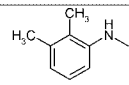
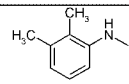
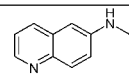
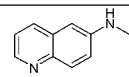
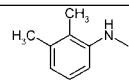
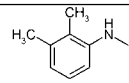
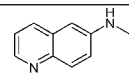
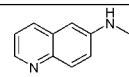
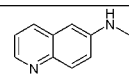
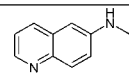
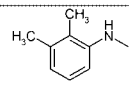
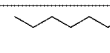
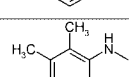
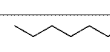
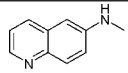
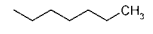
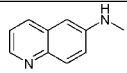
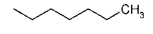
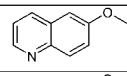
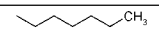
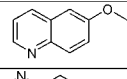
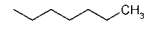
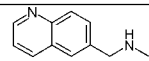
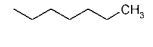
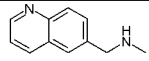
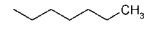
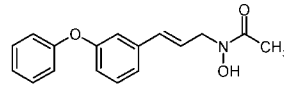
Compound				5-LO product formation in PMNL (percentage of control) at	
	R1	R2	R3	1 μM	10 μM
1		-H	-H	n.i.[a]	n.i.
2a		-H	$-C_2H_5$	76.9±23	77.7±0.8*
2b		-H	-H	n.i.	n.i.
2c		-H	$-C_2H_5$	86.3±7.7	12.3±4.4***
2d		-H	-H	n.i.	n.i.
2e		-H	$-C_2H_5$	n.i.	77.6±5.8*
2f		-H	$-C_2H_5$	80.0±9.2*	3.9±3.2***
2g		-H	-H	71.1±7.6*	14.5±1.9***
2h		-H	$-C_2H_5$	65.2±6.9*	8.9±3.2***
3a		$-CH_3$	-H	n.i.	n.i.
3b		$-CH_3$	$-C_2H_5$	n.i.	22.8±3.3***
3c		$-CH_3$	-H	n.i.	n.i.
3d		$-CH_3$	$-C_2H_5$	n.i.	12±3.6***
4a			-H	n.i.	n.i.
4b			$-C_2H_5$	53.1±9.6*	18.7±5.3***
4c			-H	n.i.	69.1±8.9*
4d			$-C_2H_5$	52.7±12.4*	3.5±1.2***
5a			-H	n.i.	50.0±4.4**
5b			$-C_2H_5$	51.8±5.8**	1.6±0.7***
6a			-H	64.3±15.4	28.9±14.3**
6b			$-C_2H_5$	84.9±14.1	30.5±2.8***

Table 1. Countinued

Compound	R1	R2	R3	5-LO product formation in PMNL (percentage of control) at 1 μM	10 μM
6c			-H	n.i.	20.1±1.9***
6d			$-C_2H_5$	27.9±5.9***	4.0±1.2***
6e			-H	91.4±8.1	10.7±2.7***
6f			$-C_2H_5$	67.4±10.5*	4.5±0.5***
6g			-H	87.6±3.8	26.7±2.2***
6h			$-C_2H_5$	57.4±11.9*	11.4±1.8***
7				3.3± 0.5***[b]	

[a] n.i. = no inhibition; [b] Inhibition at 1 μM. [c] Mean values ($n = 3-5$) and standard error estimates are given; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

μM) was supplemented in order to circumvent the necessity of $cPLA_2$-mediated endogenous substrate supply. The 5-LO inhibitor **7** (BWA4C, *N*-(3-phenoxycinnamyl)-acetohydroxamic acid)[13] was used as reference compound. As shown in Table 1, compound **1** caused no significant inhibition of 5-LO activity, but esterification of the free carboxy moiety (**2a**) led to a weak suppression of 5-LO product formation. Quinoline structures are found in several inhibitors of LT biosynthesis, although these compounds are more potent inhibitors of FLAP than of 5-LO.[14] For PPARα/γ, the mere substitution of the *o*-dimethylaniline moiety of **1** by 6-aminoquinoline leads to a total loss of activity.[10] Regarding 5-LO, exchange of the *o*-dimethylaniline of **1** by a 6-aminoquinoline (**2b**) was without effect. However, the corresponding ethyl ester **2c** caused almost complete 5-LO inhibition at 10 μM ($IC_{50} = 2.6$ μM). Linking the quinoline via oxygen (**2d**, **2e**) instead of nitrogen (**2c**) was detrimental, but insertion of an additional methylene bridge (**2f**) restored the potent inhibition ($IC_{50} = 4.2$ μM). Besides quinoline, also replacement of the *ortho*-xylene by a 2,3-dihydro-indene (**2g**, **2h**) led to efficient derivatives with IC_{50} values of 3.9 and 3.2 μM, respectively. Of interest, also the free acid (**2g**) was quite effective.

In our previous report, we showed that α-substitution of **1** with *n*-alkyl groups improved the potency at PPARα/γ.[11] Introduction of a methyl-group (**3b**) in α-position of the carboxylic acid of **1** led to potent 5-LO inhibition, again only for the esterified analogue but not for the free acid **3a**. For PPARα/γ, the replacement of *o*-dimethylaniline moiety by 6-aminoquinoline is detrimental, but concomitant α-substitution with *n*-alkyl groups restores and even enforces PPAR activation.[10,11] Regarding 5-LO, additional replacement of the *o*-dimethylaniline moiety by 6-aminoquinoline (**3d**) slightly increased the efficiency. When a phenyl moiety in α-position instead of a methyl group was introduced (compounds **4b** and **4d**), a considerable enhancement of the potency was evident, and again compounds carrying a 6-aminoquinoline were more potent versus the *o*-dimethylaniline derivatives. For example, compound **4d** inhibited 5-LO product synthesis with an IC_{50} value = 1.2 μM, which is close to the values for 1-(1-benzothiophen-2-ylethyl)-1-hydroxy-urea (zileuton, $IC_{50} = 0.5-1$ μM[15]), the most advanced 5-LO inhibitor.[1] The corresponding carboxylic acids **4a** and **4c** are not or hardly active at 10 μM. Moreover, also *n*-butyl- or *n*-hexyl-substitution in α-position was tolerated and even increased the potency leading to **5b** (*n*-butyl) and **6d** (*n*-hexyl), 6-aminoquinolines with IC_{50} values of 1.1 and 0.6 μM, respectively. Similarly, *n*-hexyl-substitution of the *o*-dimethylaniline derivative (**6b**) improves the 5-LO inhibitory potential. Apparently, the α-substitution with alkyl chains of four or six carbons enables also the corresponding free carboxylic acids (i.e., **5a**, **6a**, and **6c**) to inhibit 5-LO, possibly by masking the negative charge of the carboxylate. Replacement of the nitrogen bridge by oxygen between the pirimidinyl and the 6-aminoquinoline moieties (**6f**) or insertion of an additional methylene group (**6h**) did not significantly alter the efficiency of the esters ($IC_{50} = 1.7$ and 1.4 μM, respectively) or of the free acids (**6e** and **6g**, respectively). In Table 2, the IC_{50} values of the 10 most efficient inhibitors are summarized. Taken together, in intact stimulated PMNL compound **6d**, an α-(*n*-hexyl)-substituted

Table 2. IC_{50} Values for 5-LO of Selected Compounds Obtained in Intact PMNL

compd	IC_{50} [μM] 5-LO product synthesis
2c	2.6
2g	3.9
2h	3.2
4b	1.8
4d	1.2
5b	1.1
6a	4.1
6d	0.6
6f	1.7
6h	1.4

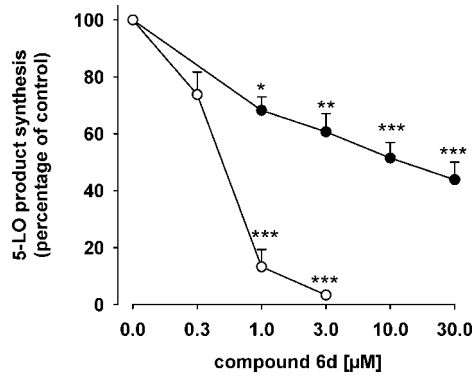


Figure 1. Inhibition of 5-LO product formation by compound **6d** in intact PMNL (-○-) and in 100000*g* supernatants of *E. coli* lysates (-●-). Error bars give the standard error of the mean ($n = 3-5$); *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table 3. Inhibition of 5-LO Product Formation by Test Compounds in 100000*g* Supernatants of *E. coli* Lysates[a]

	5-LO activity (percentage of control) at		
compd	1 μM	30 μM	IC_{50} [μM] 5-LO activity
6c	95.4 ± 1.5	52.7 ± 4.8*	34
6d	68.2 ± 4.8*	43.8 ± 6.2**	19
6e	93.7 ± 4.9	72.6 ± 4.4*	n.d.[c]
6f	79.1 ± 1.6*	59.2 ± 4.1**	n.d.[c]
6g	95.6 ± 4.7	51.0 ± 6.0**	31
6h	70.7 ± 5.0*	56.0 ± 5.9**	37
7	10.8 ± 2.6***[b]		0.2

[a] Mean values ($n = 3-4$) and standard error estimates are given; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. [b] inhibition at 1 μM. [c] n.d. = not determined.

derivative of **1**, where the *o*-dimethylaniline is replaced by a 6-aminoquinoline, is the most potent lead with an IC_{50} value of 0.6 μM.

To assess the direct interference of the compounds with 5-LO, a cell-free assay using crude human recombinant 5-LO in 100000*g* supernatants of *E. coli* lysates was applied and selected potent compounds that blocked 5-LO product synthesis in intact PMNL were evaluated. As shown in Figure 1, compound **6d** which concentration-dependently inhibited 5-LO in intact PMNL, significantly suppressed 5-LO activity in cell-free assays starting at 1 μM. However, complete 5-LO inhibition as observed in intact PMNL was not attained in the cell-free assay, not even at 30 μM, where about 45% activity still remained. A similar 5-LO inhibition pattern was observed for **6f** and **6h** (Table 3) and other derivatives tested (not shown). In contrast, compound **7** was about equipotent in the cell-free assay as compared to the cell-based test system, which is in agreement with the literature.[13,16,17]

Differences in the potencies of 5-LO inhibitors depending on the assay conditions and/or experimental settings are frequently observed.[4,12] Of interest, in the cell-free assay, the free carboxylic acids **6c**, **6e**, and **6g** blocked 5-LO activity at 30 μM almost equally as well as their corresponding esters **6d**, **6f**, and **6h**, respectively (Table 3). Possibly, the more hydrophobic ester group causes enrichment of the compounds inside the cell and/or confers the compounds an improved ability to suppress cellular 5-LO product synthesis, which does not apply to 5-LO inhibition in cell-free assays. At the moment, the molecular mode of action and the classification of the derivatives of **1** as 5-LO inhibitors is unclear. LT biosynthesis inhibitors are grouped into direct 5-LO inhibitors classified as redox-active compounds, iron-ligand inhibitors, and nonredox-type inhibitors, and into inhibitors of FLAP.[4] Redox-active 5-LO inhibitors encompass lipophilic reducing agents that uncouple the catalytic cycle of the enzyme by reducing the active site iron or by scavenging electrons/radicals.[18,19] Similarly, iron-ligand inhibitors, containing hydroxamic acid residues (e.g., compound **7**) or hydroxyurea moieties (e.g., zileuton), act at the active site by chelating and reducing the iron. For the derivatives of **1**, no redox properties or iron-chelating features are readily apparent from their chemical structure and ongoing studies exclude antioxidant properties. The nonredox-type 5-LO inhibitors compete with AA or lipid hydroperoxides for binding to 5-LO without redox properties[17] and the derivatives of compound **1** might act as nonredox-type 5-LO inhibitors. However, typical representatives out of this group (e.g., ZM-230487 (1-ethyl-6-((3-fluoro-5-(4-methoxy-3,4,5,6-tetrahydro-2*H*-pyran-4-yl)phenoxy)methyl)-2-quinolone) or L-739.010 ([1*S*,5*R*]-3-cyano-1-(3-furyl)-6-(6-[3-(3α-hydroxy-6,8-dioxabicyclo[3.2.1]octanyl)]pyridin-2-yl-methoxyl)naphthalene) differ from structural points of view and require reducing conditions (i.e., glutathione peroxidase activity) for potent inhibition of 5-LO, which is not the case for the derivatives of **1** (data not shown). Finally, FLAP inhibitors prevent the transfer of AA to 5-LO or at least interrupt the access of 5-LO toward its substrate but fail to inhibit 5-LO in cell-free assays.[20] Although FLAP inhibition by the derivatives of **1** can not be excluded and is actually favored due to the higher effectiveness of the compounds in intact cells versus cell-free assays, a direct 5-LO inhibitory effect is still obvious. Nevertheless, because quinoline structures (that improved the potency of the derivatives) govern FLAP inhibition,[14] one may speculate that there might exist some additivity (inhibition of FLAP and of 5-LO) that results in potent suppression of cellular LT formation. Moreover, the compounds could interrupt the stimulatory interaction of 5-LO with coactosin-like protein (CLP) in intact PMNL[21] or might affect phosphorylation processes that regulate cellular 5-LO activation.[3] Finally, an impairment of 5-LO-activating hydroperoxides[4] due to interference with cellular redox systems (e.g., NADPH oxidase) is conceivable.

Conclusions

Here we showed that α-substituted esters of compound **1** act as novel and potent inhibitors of 5-LO product synthesis in intact stimulated PMNL, exemplified by **6d**, with an IC_{50} value of 0.6 μM, where the *o*-dimethylaniline is replaced by a 6-aminoquinoline. Although the biochemical mechanisms of these compounds leading to the potent suppression of cellular 5-LO product synthesis are not entirely clear, the high effectiveness in intact PMNL is promising and encourages for further investigations. Future experiments aiming to resolve the detailed interference of these compounds with FLAP, the 5-LO enzyme, or other determinants of cellular 5-LO product formation (e.g., CLP, phosphorylation events), as well as studies addressing the effectiveness in vivo using animal models of inflammation, are necessary and may reveal the therapeutic potential of these derivatives.

Materials and Methods

Compounds. Compound **1** was synthesized according to D'Atri et al.[22] and the compounds **2a**−**6h** were synthesized as previously reported.[10,11] 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.[10,11]

Assay Systems. Materials. Arachidonic acid and Ca^{2+}-ionophore A23187 were from Sigma (Deisenhofen, Germany). HPLC solvents were from Merck (Darmstadt, Germany).

Cell Preparation. Human PMNL were freshly isolated from leukocyte concentrates obtained at St. Markus Hospital (Frankfurt, Germany) or 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 RT. PMNL were immediately isolated by dextran sedimentation, centrifugation on Nycoprep cushions (PAA Laboratories, Linz, Austria), and hypotonic lysis of erythrocytes as described.[23] Cells were finally resuspended in phosphate-buffered saline pH 7.4 (PBS) containing 1 mg/mL glucose and 1 mM $CaCl_2$ (PGC buffer) (purity >96−97%).

Expression of Human Recombinant 5-LO in *E. coli* and Preparation of High Speed Supernatants. *E. coli* MV1190 was transformed with pT3-5LO plasmid, and recombinant 5-LO protein was expressed at 27 °C as described.[16] Cells were lysed by incubation in 50 mM triethanolamine/HCl pH 8.0, 5 mM EDTA, soybean trypsin inhibitor (60 μg/mL), 1 mM phenylmethylsulfonyl fluoride (PMSF), and lysozyme (500 μg/mL), homogenized by sonication (3 × 15 s), and centrifuged at 100000*g* for 70 min at 4 °C. The resulting 100000*g* supernatant was immediately used for 5-LO activity assays.

Determination of 5-LO Product Formation in Intact cells. For assays of intact cells, 5×10^6 freshly isolated PMNL were resuspended in 1 mL PGC buffer. After preincubation with the test compounds for 15 min at RT, 5-LO product formation was started by addition of 2.5 μM ionophore A23187 plus 20 μM 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 of prostaglandin B_1, and 500 μL of PBS were added. Formed 5-LO metabolites were extracted and analyzed by HPLC as described.[23] 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, and 5(*S*)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid. Cysteinyl LTs C_4, D_4, and E_4 were not detected, and oxidation products of LTB_4 were not determined.

Determination of 5-LO Product Formation in Cell-Free Systems. For determination of the activity of 5-LO in 100000*g* supernatants, aliquots of the supernatants (approximately 20 μL, corresponding to 4 mL *E.coli* cell culture) were added to 1 mL of a 5-LO reaction mix (PBS, pH 7.4, 1 mM EDTA, and 1 mM ATP). Samples were preincubated with the test compounds for 10 min at 4 °C, samples were prewarmed for 30 s at 37 °C, and 2 mM $CaCl_2$ and 20 μM AA were added to start 5-LO product formation. The reaction was stopped after 10 min at 37 °C by addition of 1 mL of ice-cold methanol, and the formed metabolites were analyzed as described for intact cells.

Statistics. Data are expressed as mean ± SE. IC_{50} values, obtained from measurements at 4−5 different concentrations of the compounds, are approximations determined by graphical analysis (linear interpolation between the points between 50% activity). 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 posthoc 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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