Pirinixic Acid Derivatives as Novel Dual Inhibitors of Microsomal Prostaglandin E_2 Synthase-1 and 5-Lipoxygenase

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Dual inhibition of the prostaglandin (PG) and leukotriene (LT) biosynthetic pathway is supposed to be superior over single interference, both in terms of efficacy and side effects. Here, we present a novel class of dual microsomal PGE_2 synthase-1/5-lipoxygenase (5-LO) inhibitors based on the structure of pirinixic acid [PA, 2-(4-chloro-6-(2,3-dimethylphenylamino)pyrimidin-2-ylthio)acetic acid, compound **1**]. Target-oriented structural modification of **1**, particularly α substitution with extended *n*-alkyl or bulky aryl substituents and concomitant replacement of the 2,3-dimethylaniline by a biphenyl-4-yl-methane-amino residue, resulted in potent suppression of mPGES-1 and 5-LO activity, exemplified by 2-(4-(biphenyl-4-ylmethylamino)-6-chloropyrimidin-2-ylthio)octanoic acid (**7b**, IC_{50} = 1.3 and 1 μM, respectively). Select compounds also potently reduced PGE_2 and 5-LO product formation in intact cells. Importantly, inhibition of cyclooxygenases-1/2 was significantly less pronounced. Taken together, these pirinixic acid derivatives constitute a novel class of dual mPGES-1/5-LO inhibitors with a promising pharmacologial profile and a potential for therapeutic use.

Introduction

Prostaglandins (PGs)[a] and leukotrienes (LTs) are synthesized from arachidonic acid (AA) by the initial actions of the key enzymes cyclooxygenase (COX) and 5-lipoxygenase (5-LO), respectively.[1] Pathological conditions, such as inflammation, pain, fever, anorexia, atherosclerosis, stroke, and tumorigenesis, are related to elevated levels of PGE_2.[2] Accordingly, pharmacological inhibitors of COX isoenzymes [i.e., nonsteroidal anti-inflammatory drugs (NSAIDs) or COX-2-selective coxibs] are used to treat respective disorders.[1] However, other PGs (e.g., PGI_2 and $PGF_{2\alpha}$) are needed to maintain homeostasis, and thus, inhibition of the synthesis of these PGs by NSAIDs is likely responsible for gastrointestinal and renal side effects.[3] Although coxibs have a lower risk of gastrointestinal damage, they exert cardiovascular side effects presumably by altering the balance between PGI_2 and thromboxanes.[4]

PGE_2 is synthesized from the COX product PGH_2 by three terminal isoforms of PGE_2 synthases (PGES). Increasing evidence suggests that the microsomal PGE_2 synthase-1 (mPGES-1), which is induced by pro-inflammatory stimuli and functionally coupled to COX-2, provides elevated PGE_2 levels during inflammation, fever, and pain.[2] Thus, mPGES-1 may constitute a potential target for therapeutic intervention,[5] but only a few mPGES-1 inhibitors have been described thus far. Compound **9** [MK-886, 3-(3-(*tert*-butylthio)-1-(4-chlorobenzyl)-5-isopropyl-1*H*-indol-2-yl)-2,2-dimethylpropanoic acid, IC_{50} = 2.4 μM[6]] and structural derivatives[7] potently inhibit mPGES-1 *in vitro*, but they are afflicted to high plasma protein binding. Recently, phenanthrene imidazoles have been identified as selective and orally active mPGES-1 inhibitors (IC_{50} = 1.3 μM in whole blood[8]) that effectively relieve both pyresis and inflammatory pain in preclinical models of inflammation.[9]

LTs are important mediators of inflammatory and allergic diseases but also play roles in cardiovascular disease and cancer.[10] Anti-LT therapy is used in asthma therapy and may possess therapeutic potential also for other LT-related disorders. It was found that dual inhibition of both the PG and LT biosynthetic pathway, for example, by 2-(6-(4-chlorophenyl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1*H*-pyrrolizin-5-yl) acetic acid (licofelone), is superior over single interference, not only in terms of anti-inflammatory effectiveness but also because of a lower incidence of gastrointestinal toxicity, typically related to COX inhibition.[11,12] We have recently shown that licofelone suppresses PGE_2 formation primarily by interference with mPGES-1, whereas COX-2 was hardly affected.[13] It is speculated that drugs that selectively suppress PGE_2 formation combined with reduction of LT biosynthesis may lack the detrimental cardiovascular side effects, implying an alternative pharmacological strategy for intervention with inflammation.[5,14]

Recently, we have shown that aliphatic α substitution of pirinixic acid [PA, 2-(4-chloro-6-(2,3-dimethylphenylamino)pyrimidin-2-ylthio)acetic acid, compound **1**] enhanced both peroxisome proliferator-activated receptor (PPAR)α and PPARγ agonism[15] and enables these derivatives to inhibit cellular LT formation (IC_{50} = 0.6 μM).[16] Here, we address the effectiveness of **1** and its derivatives for inhibition of PGE_2 synthesis and interaction with mPGES-1, 5-LO, and COX-1/2. Although **1** itself failed to suppress PGE_2 formation, we present α-substi-

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[a] Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; cPGES, cytosolic prostaglandin E_2 synthase; ELISA, enzyme-linked immunosorbent assay; FLAP, 5-LO-activating protein; 12-HHT, 12(*S*)-hydroxy-5-*cis*-8,10-*trans*-heptadecatrienoic acid; IL, interleukin; 5-LO, 5-lipoxygenase; LT, leukotriene; mPGES, microsomal prostaglandin E_2 synthase; NSAID, nonsteroidal anti-inflammatory drug; PBS, phosphate-buffered saline; PPAR, peroxisome proliferator-activated receptor; PA, pirinixic acid; PMNL, polymorphonuclear leukocyte; PG, prostaglandin.

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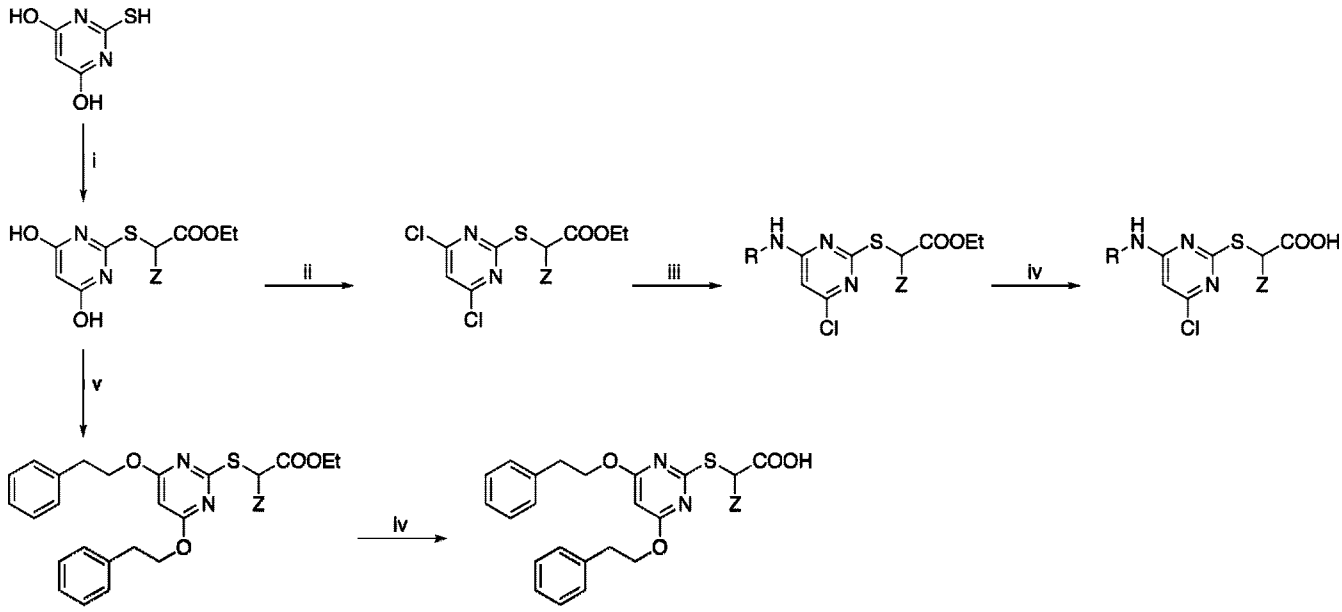
Scheme 1. General Synthesis of Compounds **5a**−**7e**[a]



[a] Reagents and conditions: (i) (Z = H), thiobarbituric acid, 2-bromoethylester (1.17 equiv), NaOH (1.08 equiv), H_2O/EtOH (1:1), 60 °C, 2 h; (Z = *n*-Bu), (Z = *n*-Hex), thiobarbituric acid, 2-bromoethylester (1.5 equiv), triethylamine (3.0 equiv), DMF, 80 °C, 1.5−3 h; (ii) $POCl_3$ (18 equiv), *N*,*N*-diethylaniline (1 equiv), RF, 2−3.5 h; (iii) R-NH_2 (1.2 equiv), triethylamine (3 equiv), EtOH, RF, 3.5−96 h; (iv) LiOH (3 equiv), EtOH, RT, 24 h; (v) (2-bromoethyl)benzene (2 equiv), K_2CO_3 (3 equiv), DMF, 80 °C, 5−9 h.

tuted derivatives as potent dual mPGES-1/5-LO inhibitors in cellular and cell-free systems.

Results and Discussion

Chemistry. The presented compounds were generally synthesized in a four-step reaction, which was modified from the synthesis of **1** published by D'Atri et al.[17] Compounds **2a**−**4** were synthesized as previously reported.[15,18] The synthesis of compounds **5a**−**7e** is presented in Scheme 1. First, the thiol group of thiobarbituric acid was etherified with the appropriate α-bromoethylester (i). The introduction of alkyl chains longer than four carbons succeeds easier with triethylamine as a conjugated base in DMF instead of NaOH in H_2O/EtOH.[17] Aromatic nucleophilic substitution with $POCl_3$ (ii) gave the chlorinated pyrimidine derivatives. In the following step, treatment with the appropriate primary amine employing triethylamine in refluxed EtOH (iii) resulted in monoamination of the pyrimidine core. These compounds were hydrolyzed with LiOH in EtOH (iv) to give the desired carboxylic acids. A side path via a Williamson-like ether synthesis (v) led to symmetrically substituted 3,5-bis-ether pyrimidine derivatives, which were hydrolyzed to the corresponding carboxylic acids as described (iv).

Screening for Dual 5-LO and mPGES-1 Inhibitors Derived from Prinixic Acid. We have previously shown that target-oriented structural modification of **1** led to potent inhibitors of 5-LO product formation in intact polymorphonuclear leukocytes (PMNL) and to a minor extent also in cell-free assays.[16] In particular, esterification of the carboxylic acid group, replacement of the 2,3-dimethylaniline by 6-aminoquinoline, and alkylation in the α position of the carboxylic acid group yielded compound **3b** (IC_{50} = 0.6 μM in intact PMNL) as the most potent derivative out of the structures **1**−**3d** (Table 1 and ref 16). Because dual inhibition of both the LT and the PG biosynthetic pathway might be advantageous over single interference,[11] potent inhibitors that dually interfere with 5-LO product and PGE_2 formation would be desirable. Accordingly, we pursued our search for improved 5-LO inhibitors with the core structure of **1** and also addressed their effectiveness for inhibition of mPGES-1. To initially screen for dual inhibitors of 5-LO and mPGES-1, compound **1** and its derivatives (at 10 μM) were analyzed for their ability to inhibit the conversion of PGH_2 to PGE_2 mediated by mPGES-1 in microsomes of IL-1β-treated A549 cells.[13] Focus was placed on derivatives that are substituted in the α position of the carboxyl group. The representatives **2d**, **2e**, **2g**, and **3a**−**3d** out of this series were already shown to inhibit 5-LO product synthesis in human PMNL, and values given in Table 1 are referenced.[16] Thus, only for novel derivatives **2f**, **2h**, and **4**−**7e**, the 5-LO inhibitory potential was assessed in the first screening round. The 5-LO inhibitor **8** [BWA4C, (*E*)-*N*-hydroxy-*N*-(3-(3-phenoxyphenyl)-allyl)acetamide][19] and the mPGES-1 inhibitor **9** were used as reference compounds, respectively.

In accordance with the literature,[6,7,13] compound **9** concentration-dependently blocked PGE_2 formation with an IC_{50} = 2.1 μM, and 26 ± 3% activity remained at a concentration of 10 μM. Compound **1** itself did not inhibit mPGES-1 activity. Introduction of one (**2a**) or two (**2b**) methyl groups or a *n*-butyl residue (**2c**) in the α position of the carboxyl group of **1** was without effect. However, elongation of the *n*-alkyl chain [*n*-hexyl (**2d**) and *n*-octyl (**2f**)] yielded potent inhibitors of mPGES-1 (Table 1). The α-phenyl-substituted **2g** was hardly active, but introduction of a naphthyl moiety (**2h**) clearly enhanced the efficacy against mPGES-1, comparable to the *n*-octyl-substituted **2f**. Together, *n*-hexyl, *n*-octyl, or naphthyl substitution of **1** in the α position leads to mPGES-1 inhibition, implying a requirement of bulky lipophilic substituents. Note that the novel derivatives **2h** and **2f**, which are carboxylic acids possessing bulky α-substituents, also efficiently inhibited 5-LO product synthesis in PMNL (Table 1), suggesting that similar SARs are applicable for both mPGES-1 and 5-LO. The effectiveness of the free carboxylic acids against 5-LO is surprising because, for interference with 5-LO product formation in PMNL, the corresponding esters have been found to be generally superior

Table 1. Inhibition of PGE_2 Formation in Microsomal Preparations of A549 Cells and Inhibition of 5-LO Product Formation in PMNL by Test Compounds[d]

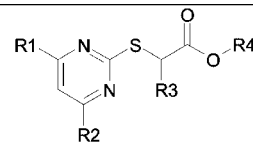
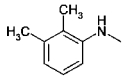
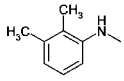
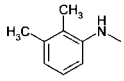
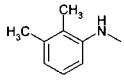
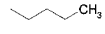
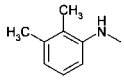
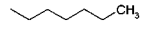
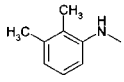
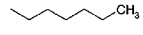
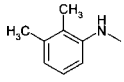
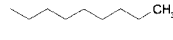
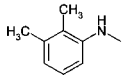
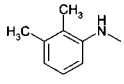
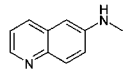
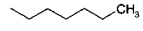
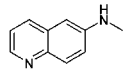
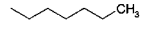
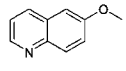
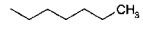
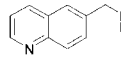
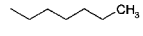
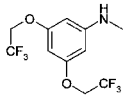
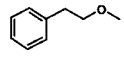
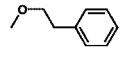
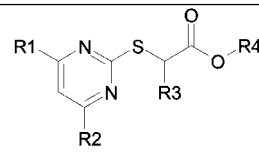
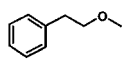
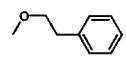
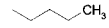
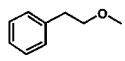
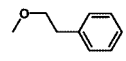
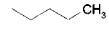
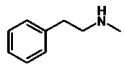
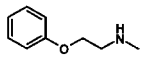
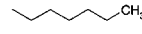
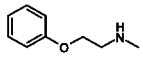
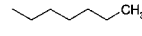
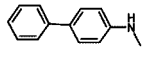
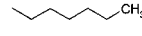
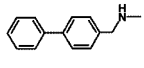
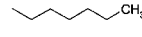
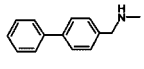
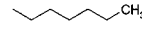
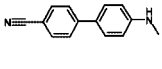
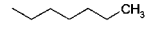
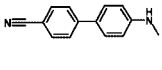
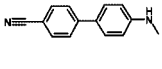
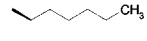
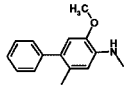
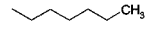
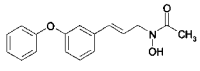
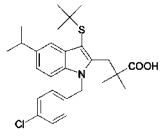
comp.	 R1	R2	R3	R4	cell-free mPGES-1 activity (percentage of control) at 10 μM	PMNL 5-LO activity (percentage of control) at 10 μM
1		-Cl	-H	-H	n.i.[a]	n.i.[b]
2a		-Cl	$-CH_3$	-H	n.i.	n.i.[b]
2b		-Cl	$-(CH_3)_2$	-H	83.8 (± 7.4)	n.i.[b]
2c		-Cl		-H	n.i.	n.i.[b]
2d		-Cl		-H	31.9 (± 5.6)***	28.9 (± 14.3)***b
2e		-Cl		$-C_2H_5$	n.i.	30.5 (± 2.8)***b
2f		-Cl		-H	29.8 (± 7.1)***	7.0 (± 2.3)***
2g		-Cl		-H	74.6 (± 1.9)	n.i.[b]
2h		-Cl		-H	39.6 (± 5.2)***	16.8 (± 9.6)***
3a		-Cl		-H	59.0 (± 6.5)**	20.1 (± 1.9)***b
3b		-Cl		$-C_2H_5$	39.1 (± 5.8)***	4.0 (± 1.2)***b
3c		-Cl		-H	67.4 (± 7.3)*	10.7 (± 2.7)***b
3d		-Cl		-H	85.7 (± 8.9)	26.7 (± 2.2)***b
4		-Cl		-H	26.7 (± 1.8)***	8.0 (± 4.0)***
5a			-H	-H	66.4 (± 2.4)*	40.6 (± 10.0)***

Table 1. Continued

comp.	 R1	R2	R3	R4	cell-free mPGES-1 activity (percentage of control) at 10 μM	PMNL 5-LO activity (percentage of control) at 10 μM
5b				-H	43.1 (± 5.4)***	2.7 (± 0.2)***
5c				$-C_2H_5$	n.i.	80.1 (± 9.1)
6a		-Cl		-H	70.0 (± 9.4)	16.07 (± 6.3)***
6b		-Cl		-H	79.9 (± 6.2)	28.0 (± 8.0)***
6c		-Cl		$-C_2H_5$	80.7 (± 8.0)	37.1 (± 4.9)***
7a		-Cl		-H	21.7 (± 2.9)***	4.1 (± 1.1)***
7b		-Cl		-H	21.8 (± 4.2)***	3.0 (± 2.3)***
7c		-Cl		$-C_2H_5$	n.i.	80.2 (± 9.9)
7d		-Cl		-H	26.1 (± 8.0)***	2.7 (± 0.8)***
7d(S)		-Cl		-H	23.0 (± 5.9)***	2.8 (± 1.7)***
7d(R)		-Cl		-H	36.8 (± 4.7)***	2.8 (± 1.5)***
7e		-Cl		-H	24.9 (± 1.2)***	3.1 (± 0.6)***
8					n.i.	3.3 (± 0.5)***c
9					25.5 (± 2.8)***	n.i.

[a] n.i. = no inhibition. [b] Published by Werz et al.[16] [c] Inhibition at 1 μM. [d] Mean values ($n = 3-4$) and standard error estimates are given: (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$.

over the free acids.[16] Here, we find that the need for esterification was abolished by insertion of long and bulky alkyl/aryl residues in the α position of the carboxylic group, reflected by the high efficacy of the α-naphthyl derivative **2h** and the α-(*n*-octyl)-analogue **2f** against 5-LO.

Replacement of the 2,3-dimethylphenyl residue by a quinoline moiety (**3a**−**3d**), regardless of the bridging atom (N or O), was shown to significantly improve inhibition of 5-LO product formation.[16] Although this derivatization did not enhance the potency of the α-(*n*-hexyl)-substituted free acids **3a**, **3c**, and

3d toward mPGES-1, compound **3b**, the esterified analogue of **3a**, was quite efficient and remarkably superior over its 2,3-dimethylphenyl analogue **2e**. Therefore, the presence of a quinoline residue apparently overcomes the necessity of the free carboxylic group, whereas all other esters tested were rather poor inhibitors of mPGES-1. For example, **2e**, the ethyl ester of **2d** (that potently inhibited mPGES-1), failed to suppress PGE_2 formation, suggesting that the free carboxylic group governs mPGES-1 inhibition. Why the ester **3b** was superior over the free acid as inhibitor of mPGES-1 is not readily understood and requires more detailed analysis.

Also, the exchange of the 2,3-dimethylaniline by more bulky lipophilic substituents at C6 of the pyrimidine, for example, by 3,5-bis(2,2,2-trifluoroethoxy)-aniline (**4**), led to derivatives that were similarly active on mPGES-1 as compared to **2d**. Of interest, the carboxylic acid **4** also potently inhibited 5-LO product synthesis. Hence, bulky lipohilic substituents not only in the α position but also linked to the pyrimidine ring may allow the free acid analogues to inhibit cellular 5-LO product formation. Along these lines, replacement of the 2,3-dimethylaniline moiety and the chlorine at the pyrimidine ring of **1** by phenylethoxy residues led to compound **5a** and its α-butyl analogue **5b**, free acids that moderately (**5a**) or potently (**5b**) blocked 5-LO product synthesis in PMNL, respectively. Both compounds also inhibited mPGES-1, and again, the corresponding ester of **5b**, namely, compound **5c**, was not (mPGES-1) or hardly (5-LO) active.

In view of the SARs regarding mPGES-1 apparent thus far, bulky lipophilic substituents at C6 of the pyrimidine ring could basically increase the efficacy. Whereas exchange of the 2,3-dimethylaniline by phenethylamine (**6a**) or phenoxyethylamine (**6b** or **6c**) failed to increase the potency versus **2d**, introduction of a biphenyl-4-amine (**7a**) or a biphenyl-4-methane amine moiety (**7b**) yielded derivatives that potently inhibited mPGES-1 as well as 5-LO. The corresponding esters (exemplified by **7c**) failed to interfere with mPGES-1 and also were hardly active against 5-LO. Moreover, substitutions of the biphenyl-4-amino moiety were tolerated, because introduction of a cyano group in position 4′ (**7d**) or methyl and methoxy substitutents at the biphenyl (**7e**) did not hamper the interference with either mPGES-1 or 5-LO. Finally, we investigated whether or not the absolute configuration of the α-C atom next to the carboxyl group influences the potencies of the compounds. The two enantiomeric forms of the *n*-hexyl-substituted derivative **7d**, namely, **7d**(*R*) and **7d**(*S*) were separated and tested for inhibition of 5-LO and mPGES-1. Neither for inhibition of 5-LO (intact cells or cell-free assay) nor for mPGES-1 was a marked discrepancy in the efficacies (mPGES-1, $IC_{50} = 1.6$ and 2.1 μM, respectively, and cell-free 5-LO, $IC_{50} = 0.8$ and 1.5 μM, respectively) determined, implying that the absolute configuration is essentially negligible. Taken together, two structural modifications of the parental inactive compound **1**, namely, (I) introduction of an *n*-hexyl residue in the α position and (II) replacement of the 2,3-dimethylaniline at the C6 of the pyrimidine ring by a biphenyl-4-yl-methanamine residue yield efficient dual mPGES-1/5-LO inhibitors, including **7a**, **7b**, **7d**, and **7e**, with about equal efficacies for both enzymes.

Detailed Analysis of Selected Test Compounds on mPGES-1, 5-Lipoxygenase, and Cyclooxygenases. As stated above and shown in Table 2, there is obviously a strong correlation between the efficiency against mPGES-1 and 5-LO, and all eight representatives chosen block both enzymes similarly well with comparable IC_{50} values in the range of 1.3−5.6 μM for mPGES-1 and in the range of 0.4−5 μM for 5-LO in PMNL. Compound **7b** turned out to be the most potent mPGES-1 inhibitor, with an $IC_{50} = 1.3$ μM, being even somewhat superior over **9** [$IC_{50} = 2.1$ (Table 2) and 2.4 μM[6]]. Compound **7d** was the most active inhibitor of 5-LO in intact PMNL and purified 5-LO ($IC_{50} = 0.4$ and 1.5 μM, respectively), with similar potency as 1-(1-(benzo[*b*]thiophen-2-yl)ethyl)-1-hydroxyurea (zileuton, Zyflo), the most advanced 5-LO inhibitor under comparable conditions ($IC_{50} = 0.5$ to 1 μM[20]). Importantly, the reported discrepancy between the potency regarding 5-LO inhibition in cell-based assays (high efficacy) and cell-free systems (low efficacy) that was originally apparent for the ester **3b** and other esterified derivatives of **1**[16] is not evident for the free acids. Thus, the α-(*n*-hexyl)-substituted acids bearing a bulky lipophilic residue at C6 of the pyrimidine ring also potently inhibit 5-LO in cell-free assays, with IC_{50} values in the range of 1.5−6.5 μM, and can thus be designated direct 5-LO inhibitors.

Ideally, the preferred pharmacological dynamics of a lead compound for intervention with chronic inflammatory diseases, CVD, or cancer without typical side effects of NSAIDs and coxibs could comprise a potent and dually suppressive effect on mPGES-1 and 5-LO without inhibiting COX isoenzymes.[13] Accordingly, selected potent dual mPGES-1/5-LO inhibitors from the first screening round were investigated for their pharmacological profile in more detail. Because lipophilic acids, such as **1** and its derivatives mimicking AA, are likely to act on COX enzymes and because the active lead compounds inhibit the COX-related mPGES-1 and 5-LO about equally well, it appeared reasonable to address if the compounds may also interfere with COX-1/2. The effects of selected compounds on the activities of isolated ovine COX-1 and human recombinant COX-2 [using the COX metabolite 12(*S*)-hydroxy-5-*cis*-8,10-*trans*-heptadecatrienoic acid (12-HHT) as a biomarker[21]] were determined. With the exception of compounds **2h** and **4**, all selected derivatives were only moderate inhibitors of COX-1 and COX-2, with IC_{50} values > 10 μM. In control experiments, 2-[1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]acetic acid (indomethacin, 10 μM) suppressed the activity of COX-1 and 4-(5-(4-methylphenyl)-3-(trifluoromethyl)pyrazol-1-yl)benzenesulfonamide (celecoxib, 5 μM) inhibited the activity of COX-2 with 19 ± 2 and 21 ± 3% remaining activity, respectively. Therefore, we conclude that COX inhibition by the lead compounds is markedly less pronounced as compared to interference with 5-LO and mPGES-1.

Effectiveness of the PA Derivatives on PGE_2 Formation in Intact A549 Cells. In contrast to the convenient cell-based assay applied for the evaluation of 5-LO inhibitors, assessment of mPGES-1 inhibitors in intact cell assays is laborious.[13] Hence, we analyzed only select compounds (i.e., **2d**, **4**, and **7b**) for inhibition of PGE_2 formation in IL-1β-stimulated A549 cells. For PGE_2 biosynthesis in intact cells, phospholipases A_2 liberate AA as a substrate for COX isoenzymes to produce PGH_2 that is eventually transformed by selective PG or thromboxane synthases to various structurally related eicosanoids. In IL-1β-treated A549 cells, PGH_2 is provided by highly expressed COX-2 and converted by mPGES-1,[22] whereas COX-1 could not be detected.[23] After 48 h of treatment with IL-1β and preincubation (10 min) with test compounds, cells were activated via massive intracellular Ca^{2+} influx using 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]. Simultaneously, exogenous AA [1 μM AA and 18.4 kBq [^{3}H]AA] was provided to initiate PGH_2 formation and

Table 2. IC_{50} Values for mPGES-1 in Microsomal Preparations of A549 Cells, 5-LO Activity in PMNL, and Purified 5-LO in a Cell-Free Assay[c]

comp.	structure	mPGES-1 (IC_{50} [μM]) cell-free	5-LO (IC_{50} [μM]) PMNL	5-LO (IC_{50} [μM]) pur. 5-LO	COX (remaining activity at 10 μM) COX-1	COX (remaining activity at 10 μM) COX-2
2h	R1 = 2,3-dimethylaniline R2 = naphthyl R3 = -H	5.1	5.0	n.d.[a]	42.2 (±11.4)***	32.5 (± 7.3)***
2d	R1 = 2,3-dimethylaniline R2 = *n*-hexyl R3 = -H	3.9	4.1	6.5	75.2 (± 8.9)	62.0 (± 5.6)*
3b	R1 = 6-aminoquinoline R2 = *n*-hexyl R3 = -C_2H_5	5.6	0.6	19.0	69.5 (± 1.9)	61.7 (± 9.3)*
4	R1 = 3,5-bis(2,2,2-trifluoroethoxy)aniline R2 = *n*-hexyl R3 = -H	2.6	1.5	3.0	40.0 (± 8.9)***	70.4 (± 9.3)
7a	R1 = biphenyl-4-amine R2 = *n*-hexyl R3 = -H	1.6	0.7	1.5	52.3 (± 0.9)**	57.1 (± 5.1)**
7b	R1 = biphenyl-4-yl-methan-amine R2 = *n*-hexyl R3 = -H	1.3	1.0	2.0	67.3 (± 1.2)	61.3 (± 3.7)**
7d	R1 = 4'-cyanobiphenyl-4-ylamine R2 = *n*-hexyl R3 = -H	1.7	0.4	1.5	66.0 (± 8.3)*	61.2 (± 3.6)**
7e	R1 = 5-methoxy-2-methyl-biphenyl-4-amine R2 = *n*-hexyl R3 = -H	2.1	0.5	2.0	71.2 (± 1.0)	53.7 (± 3.8)**
8	*N*-(3-phenoxycinnamyl)-acetohydroxamic acid	n.i.[b]	0.11	0.16	n.d.	n.d.
9	3-[1-(4-chlorobenzyl)-3-t-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid	2.1	0.09	n.i.[b]	n.d.	n.d.

[a] n.d. = not determined. [b] n.i. = no significant inhibition at 10 μM. [c] Inhibition of isolated ovine COX-1 and human recombinant COX-2 by selected compounds given as remaining activity at 10 μM. Mean values ($n = 3$) and standard error estimates are given: (∗) $p < 0.05$, (∗∗) $p < 0.01$, and (∗∗∗) $p < 0.001$.

circumvent the need for release of endogenous AA by phospholipases. ^{3}H-labeled PGE_2 was separated by RP-HPLC from other eicosanoids and quantified by liquid scintillation counting. As expected, PGE_2 formation was almost completely suppressed by the COX inhibitors indomethacin (10 μM, data not shown) or celecoxib (5 μM) (Figure 1). Inhibition of mPGES-1 in intact A549 cells by **9** (33 μM) reduced PGE_2 formation by only 63%. Compounds **2d** and **7b** concentration-dependently inhibited PGE_2 formation, with apparent IC_{50} values of 12 and 6 μM, respectively (Figure 1). However, inhibition of PGE_2 formation already started at effective concentrations ≥1−3 μM, but neither **2d**, **4**, or **7b** nor the mPGES-1 inhibitor **9** completely inhibited PGE_2 synthesis. Compound **4** was markedly less potent, and inhibition of PGE_2 formation started first at 10 μM, with the apparent IC_{50} value being >30 μM (Figure 2).

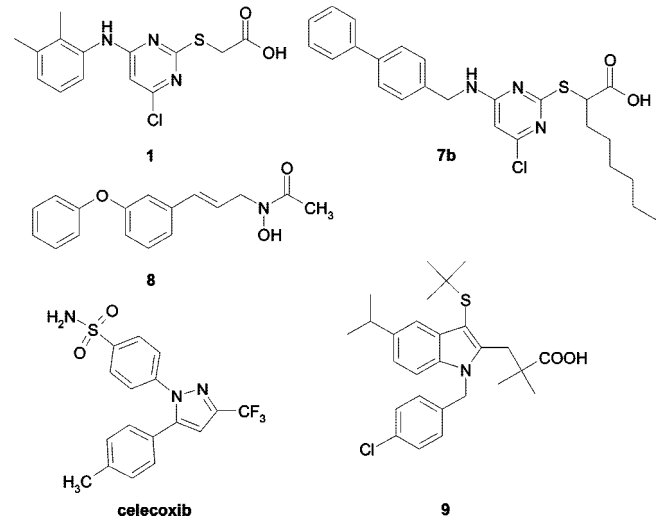


Figure 1. Chemical structures of selected inhibitors.

Conclusions

Here, we present the design of compounds that combine a potent suppression of PGE_2 formation by inhibiting mPGES-1 with a potent 5-LO inhibitory action, leading to reduced levels of LTs, but lack pronounced interference with both COX-1 and COX-2. In particular, the biphenylic derivatives of **1** (e.g., **7b**) were most potent, but also **2d** and **4** are efficient dual inhibitors of mPGES-1 and 5-LO in cell-free assay and cell-based systems. However, in contrast to the COX inhibitors celecoxib and indomethacin, PGE_2 formation was not completely suppressed in A549 cells by the derivatives of **1** as well as by **9**, with 30−40% PGE_2 still remaining, probably related to the action of other PGE_2 synthases not affected by the compounds. Dual inhibitors of COX/5-LO are superior over compounds interfering

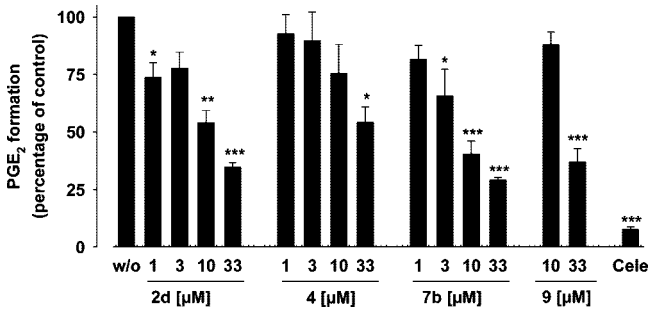


Figure 2. Effects of test compounds on the formation of PGE_2 in intact A549 cells. A549 cells (4×10^6/mL) were pre-incubated with test compounds or vehicle (DMSO) for 10 min, and then cellular PGE_2 synthesis was elicited by addition of 2.5 μM Ca^{2+} ionophore A23187 plus 1 μM AA and [^{3}H]AA (18.4 kBq). After 15 min at 37 °C, formed [^{3}H]PGE_2 was analyzed by RP-HPLC and liquid scintillation counting as described in the Materials and Methods. Data are given as mean ± standard error (SE). $n = 3$. (*) $p < 0.05$, (**) $p < 0.01$, or (***) $p < 0.001$ versus vehicle (0.1% DMSO) control. ANOVA + Tukey HSD posthoc tests. Cele = celecoxib, 5 μM.

with only COX enzymes (i.e., NSAIDs and coxibs) in terms of anti-inflammatory effectiveness associated with a lower incidence of gastrointestinal and cardiovascular toxicity.[11] This might be attributable to the accompanied suppression of pro-inflammatory LTs,[24] which significantly contribute to gastric epithelial injury and atherogenesis.[10] For example, licofelone that blocks mPGES-1, LT formation, and COX-1 without inhibiting COX-2[13] exhibits a significantly superior gastric tolerability and a lower incidence of ulcers compared to (*S*)-2-(6-methoxynaphthalen-2-yl)propanoic acid (naproxen) in healthy humans,[25] and in contrast to COX-2-selective inhibitors, licofelone reduced neointimal formation and inflammation in an atherosclerotic model.[26] To our knowledge, no dual mPGES-1/5-LO inhibitor has been reported, although MK-886 and licofelone inhibit mPGES-1[6,13] and the 5-LO-activating protein,[27,28] also leading to suppression of PGE_2 and LT formation. Future experiments aiming to resolve the detailed interference of these compounds presented here with mPGES-1 and the 5-LO enzyme as well as studies addressing the effectiveness *in vivo* using animal models of inflammation, fever, pain, cancer, or atherosclerosis are necessary and may reveal the therapeutic potential of PA derivatives for intervention with mPGES-1- and 5-LO-related diseases.

Materials and Methods

Compounds and Chemistry. Compound **1** was purchased from Sigma-Aldrich (Deisenhofen, Germany). The structures of previously reported compounds **2a**−**4** were confirmed by ^{1}H and ^{13}C NMR, as well as by mass spectrometry (ESI); the purity (>98%) was checked by combustion analysis as described.[15,18]

Compounds **5a**−**7e** were synthesized as described in Scheme 1. The synthesis is modified from the method reported from D'Atri et al.[17] All commercial chemicals and solvents are of reagent grade and were used without further purification, unless otherwise specified. ^{1}H and ^{13}C NMR spectra were measured in DMSO-d_6 or $CDCl_3$ on a Bruker ARX 300 (^{1}H NMR) and AC 200 E (^{13}C NMR) spectrometer. Chemical shifts are reported in parts per million (ppm) using tetramethylsilane (TMS) as an internal standard. Mass spectra were obtained on a Fissous Instruments VG Platform 2 spectrometer measuring in the positive- or negative-ion mode (ESI−MS system). Elemental analysis has been performed by the Microanalytical Laboratory of the Institute of Organic Chemistry and Chemical Biology, Goethe-University Frankfurt, on a Foss Heraeus CHN-O-rapid elemental analyzer (for details see the Supporting Information).

The synthetic procedure is exemplified by the following description of the synthesis of key compounds **7b** and **7d**.

Step (i) (Z = *n*-Hexyl). 2-Thiobarbituric acid (1 equiv) was dissolved under heating in DMF/triethylamine (3 equiv), and ethyl-2-bromooctyl acetate (1.5 equiv) was added dropwise. After 2 h, the reaction mixture was quenched with 4 parts of water and extracted with ethyl acetate. The residue obtained from the organic phase was recrystallized from ethyl acetate/*n*-hexane to give ethyl 2-(4,6-dihydroxypyrimidin-2-ylthio)octanoate (50%).

Step (ii). To a solution of the thioether resulting from step (i) (1 equiv) in $POCl_3$ (18 equiv), *N*,*N*-diethylaniline (1 equiv) was added. After refluxing the solution at 110 °C for 3.5 h, the excessive $POCl_3$ was distilled *in vacuo* and the oily residue was poured upon crushed ice. The aqueous solution was extracted with ethyl acetate, and combined organic layers were washed with diluted HCl, saturated $NaHCO_3$ solution, and brine. The organic layer was dried over $MgSO_4$, filtered, and evaporated under reduced pressure. Purifying the resulting oil by column chromatography (*n*-hexane/ethyl acetate) gave ethyl 2-(4,6-dichloropyrimidin-2-ylthio)octanoate (85%).

Step (iii). A mixture of the chlorinated pyrimidine (1 equiv) obtained from step (ii), the appropriate aniline/amine derivate (1.05 equiv), triethylamine (3 equiv), and EtOH was prepared and heated under reflux (**7b**-ester, 6 h; **7d**-ester, 96 h). The reaction mixture was evaporated under reduced pressure. The residue was dissolved in ethyl acetate, and the organic layer was extracted with diluted HCl, saturated $NaHCO_3$ solution, and brine. After drying over $MgSO_4$, the solvent was evaporated under reduced pressure and the residue was purified by column chromatography (*n*-hexane/ethyl acetate) to give ethyl 2-(4-(biphenyl-4-ylmethylamino)-6-chloropyrimidin-2-ylthio)octanoate (**7b**-ester, 40%) and ethyl 2-(4-chloro-6-(4′-cyanobiphenyl-4-ylamino)pyrimidin-2-ylthio)-octanoate (**7d**-ester, 60%), respectively.

Step (iv). The respective ester (1 equiv) from step (iii) was dissolved in EtOH, and LiOH (3 equiv) was added. After stirring for 24 h at room temperature, EtOH was removed and the residue was dissolved in water (under heating, if necessary, low amounts of MeOH were added). This solution was acidified with diluted hydrochloric acid, and the precipitate was filtered, washed to neutrality with water and then with *n*-hexane. Recrystallization from *n*-hexane/ethyl acetate gave the carboxylic acids.

2-(4-(Biphenyl-4-ylmethylamino)-6-chloropyrimidin-2-ylthio)octanoic Acid (7b). mp = 110 °C. ^{1}H NMR (300.13 MHz, $(CD_3)_2SO$) δ: 0.77 (t, 3H, CH_3−Hex, $J = 6.3$ Hz), 1.14−1.28 (m, 8H, CH_2−Hex), 1.66−1.84 (m, 2H, CH_2−Hex), 4.23 (t, 1H, S−CH, $J = 7.1$ Hz), 4.49 (dd, 1H, CH_2−NH, $J_1 = 5.4$ Hz, $J_2 = 15.5$ Hz), 4.65 (dd, 1H, CH_{2B}−NH, $J_1 = 5.6$ Hz, $J_2 = 15$ Hz), 6.31 (s, 1H, Pyr−5H), 7.31−7.47 (m, 5H, Ph−H + Ph′−H), 7.61 (d, 2H, Ph−3H + Ph−5H, $J = 1.8$ Hz), 7.64 (d, 2H, Ph′−2H + Ph−6H, $J = 1.2$ Hz), 8.39 (t,1H, −NH, $J = 4.8$ Hz). ^{13}C NMR (75.45 MHz, $(CD_3)_2SO$) δ: 13.80 (CH_3−Hex), 21.89 (CH_2−Hex), 26.50 (CH_2−Hex), 28.13 (CH_2−Hex), 30.95 (CH_2−Hex), 31.39 (CH_2−Hex), 43.29 (CH_2−NH), 47.26 (S−CH), 99.54 (Pyr−C_5), 126.50 (2C, Ph−C_3 + −C_5), 126.67 (2C, Ph′−C_2 + −C_6), 127.32 (Ph′−C_4), 127.89 (2C, Ph−C_2 + −C_6), 128.85 (2C, Ph′−C_3 + −C_5), 138.04 (Ph−C_4), 138.91 (Ph′−C_1), 139.82 (Ph−C_1), 156.57 (Pyr−C_6), 162.40 (Pyr−C_4), 169.76 (Pyr−C_2), 172.72 (*C*OOH). MS (ESI+): *m/e* 470.1 $[M + H]^+$. Anal. Calcd ($C_{25}H_{28}ClN_3O_2S$): C, H, N. Yield: 80%.

2-(4-Chloro-6-(4′-cyanobiphenyl-4-ylamino)pyrimidin-2-ylthio)octanoic Acid (7d). mp = 111 °C. ^{1}H NMR (300.13 MHz, $(CD_3)_2SO$) δ: 0.77 (t, 3H, CH_3−Hex, $J = 6.5$ Hz), 1.17−1.36 (m, 8H, CH_2−Hex), 1.77−1.94 (m, 2H, CH_2−Hex), 4.37 (t, 1H, S−CH, $J = 7.1$ Hz), 6.56 (s, 1H, Pyr−5H), 7.70−7.77 (m, 4H, Ph−H), 7.85−7.92 (m, 4H, Ph′−H), 10.13 (s,1H, −NH). ^{13}C NMR (75.45 MHz, $(CD_3)_2SO$) δ: 13.77 (CH_3−Hex), 21.87 (CH_2−Hex), 26.49 (CH_2−Hex), 28.08 (CH_2−Hex), 30.93 (CH_2−Hex), 31.61 (CH_2−Hex), 47.13 (S−CH), 101.44 (Pyr−C_5), 109.50 (Ph′−C_4), 118.88 (−CN), 120.77 (2C, Ph−C_2 + −C_6), 126.92 (2C, Ph′−C_2 + −C_6), 127.50 (2C, Ph−C_3 + −C_5), 132.80 (2C, Ph′−C_3 + −C_5), 139.27 (Ph′−C_1), 143.97 (Ph−C_1), 157.48 (Pyr−C_6), 160.43

(Pyr−C_2), 170.02 (Pyr−C_4), 172.38 (*C*OOH). MS (ESI+): *m/e* 481.0 $[M + H]^+$. Anal. Calcd ($C_{25}H_{25}ClN_4O_2S$): C, H, N. Yield: 60%.

Assay Systems. Materials. DMEM/high glucose (4.5 g/L) medium, penicillin, streptomycin, and trypsin/EDTA solution were obtained from PAA (Coelbe, Germany). PGH_2 was obtained from Larodan (Malmö, Sweden). 11β-PGE_2, PGB_1, MK-886, human recombinant COX-2, and ovine isolated COX-1 were obtained from Cayman Chemical (Ann Arbor, MI). [5,6,8,9,11,12,14,15-^{3}H] AA ([^{3}H]AA) was obtained from BioTrend Chemicals GmbH (Cologne, Germany). Ultima Gold XR was obtained from Perkin-Elmer (Boston, MA). All other chemicals were obtained from Sigma-Aldrich (Deisenhofen, Germany), unless stated otherwise.

Cells and Cell Viability Assay. Blood cells were freshly isolated from leukocyte concentrates obtained at the Blood Center of the University Hospital Tuebingen (Germany) as described.[29] In brief, venous blood was taken from healthy adult donors that did not take any medication for at least 7 days, and leukocyte concentrates were prepared by centrifugation (4000*g*, 20 min, 20 °C). Cells were immediately isolated by dextran sedimentation and centrifugation on Nycoprep cushions (PAA, Coelbe, Germany). For incubations with solubilized compounds, methanol or DMSO was used as vehicle, never exceeding 1% (v/v). PMNL were immediately isolated from the pellet after centrifugation on Nycoprep cushions, and hypotonic lysis of erythrocytes was performed as described.[30] Cells were finally resuspended in phosphate-buffered saline (PBS) at pH 7.4, containing 1 mg/mL glucose and 1 mM $CaCl_2$ (PGC buffer) (purity > 96−97%).

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 units/mL), and streptomycin (100 μg/mL) at 37 °C and 5% CO_2. After 3 days, confluent cells were detached using 1× trypsin/EDTA solution and reseeded at 2×10^6 cells in 20 mL of medium. Cell viability was measured using the colorimetric thiazolyl blue tetrazolium bromide (MTT) dye reduction assay as described.[31] A549 cells (4×10^4 cells/100 μL of medium) were plated into a 96-well microplate and incubated at 37 °C and 5% CO_2 for 16 h. Then, test compounds or solvent (DMSO) 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 sodium dodecylsulfate [10% (w/v), in 20 mM HCl], and the absorbance of each sample was measured at 595 nm relative to that of vehicle (DMSO)-treated control cells using a multiwell scanning spectrophotometer (Victor^3 plate reader, Perkin-Elmer, Rodgau-Juegesheim, Germany). Compounds **1**, **2d**, **4**, and **7b** did not significantly reduce cell viability within 5 h at 10 μM (data not shown), excluding possible acute cytotoxic effects of the compound in the cellular 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 of PGC buffer. After pre-incubation with the test compounds (15 min, RT), 5-LO product formation was started by addition of 2.5 μM Ca^{2+}-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.[30] 5-LO product formation is expressed as nanograms of 5-LO products per 10^6 cells, which includes LTB_4 and its all-*trans* isomers, and 5(*S*)-hydro(p-ero)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.

Expression and Purification of Human Recombinant 5-LO from *Escherichia coli.* *E. coli* MV1190 was transformed with pT3-5LO plasmid, and recombinant 5-LO protein was expressed at 27 °C as described.[32] Cells were lysed by incubation in 50 mM triethanolamine/HCl at 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* supernatants were applied to an ATP-agarose column (Sigma A2767, Deisenhofen, Germany), and the column was eluted as described previously.[33] Partially purified 5-LO was immediately used for *in vitro* activity assays.

Determination of 5-LO Activity of Semi-purified 5-LO. Partially purified 5-LO was added to 1 mL of a 5-LO reaction mix (PBS at pH 7.4, 1 mM EDTA, and 1 mM ATP). Samples were pre-incubated with the test compounds for 10 min at 4 °C and 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 the addition of 1 mL ice-cold methanol, and the formed metabolites were analyzed as described for intact cells.

Induction of mPGES-1 in A549 Cells and Isolation of Microsomes. Preparation of A549 cells was performed as described.[22] In brief, cells (2×10^6 cells) in 20 mL of DMEM/high glucose (4.5 g/L) medium containing FCS (2%, v/v) were incubated for 16 h at 37 °C and 5% CO_2. Subsequently, the culture medium was replaced by fresh medium; 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 homogenization buffer (0.1 M potassium phosphate buffer at pH 7.4, 1 mM PMSF, 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 10000*g* for 10 min and 174000*g* for 1 h at 4 °C. The pellet (microsomal fraction) was resuspended in 1 mL of homogenization buffer, and the protein concentration was determined by the Coomassie protein assay.

Determination of PGE_2 Synthase Activity in Microsomes of A549 Cells. Microsomal membranes of A549 cells were diluted in potassium phosphate buffer (0.1 M, pH 7.4) containing 2.5 mM glutathione (100 μL total volume), and test compounds or vehicle (DMSO) was added. After 15 min, PGE_2 formation was initiated by the addition of PGH_2 (20 μM, final concentration). After 1 min at 4 °C, the reaction was terminated with 100 μL of stop solution (40 mM $FeCl_2$, 80 mM citric acid, and 10 μM of 11β-PGE_2), and PGE_2 was separated by solid-phase extraction on reversed-phase (RP) C18 material using acetonitrile (200 μL) as an eluent and analyzed by RP-HPLC [30% acetonitrile aqueous + 0.007% TFA (v/v), Nova-Pak C18 column, 5 × 100 mm, 4 μm particle size, flow rate of 1 mL/min], with UV detection at 195 nm. 11β-PGE_2 was used as an internal standard to quantify PGE_2 product formation by integration of the area under the peaks.

Determination of PGE_2 Formation in Intact A549 Cells. A549 cells (2×10^6), treated with IL-1β for 72 h as described above, were resuspended in 0.5 mL of PBS containing 1 mM $CaCl_2$ and pre-incubated with the indicated compounds at 37 °C for 10 min, and PGE_2 formation was started by the addition of Ca^{2+}-ionophore A23187 (2.5 μM), AA (1 μM), and [^{3}H]AA (18.4 kBq). The reaction was stopped after 15 min at 37 °C, and the samples were put on ice. After centrifugation (800*g*, 5 min, 4 °C), the supernatant was acidified to pH 3 by the addition of citric acid (20 μL, 2 M) and the internal standard 11β-PGE_2 (2 nmol) was added. PGE_2 was separated by solid-phase extraction and RP-HPLC as described above. The amount of 11β-PGE_2 was quantified by integration of the area under the eluted peaks. For quantification of radiolabeled PGE_2, fractions (0.5 mL) were collected and mixed with Ultima Gold XR (2 mL) for liquid scintillation counting in a LKB Wallac 1209 Rackbeta liquid scintillation counter.

Activity Assays of Isolated COX-1 and COX-2. Inhibition of the activities of isolated ovine COX-1 and human COX-2 was performed as described.[21,34] Although the purified COX-1 is not of human origin, ovine COX-1 is generally used for inhibitor studies when examining the effectiveness of compounds on the activity of isolated COX-1 enzyme.[34] 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 at pH 8, 5 mM glutathione, 5 μM hemoglobin, and 100 μM EDTA at 4 °C and pre-incubated with the test compounds for 5 min. Samples were prewarmed for 60 s at 37 °C, and AA (5 μM for COX-1 and 2 μM

for COX-2) was added to start the reaction. After 5 min at 37 °C, the COX product 12-HHT was extracted and then analyzed by HPLC as described.[29]

Statistics. Data are expressed as mean ± SE. The IC_{50} values were analyzed using GraphPad Prism. 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. A p value of <0.05 (*) was considered significant.

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Supporting Information Available: Chemical synthesis and routine analytics by ^1H and ^{13}C NMR, mass spectrometry (ESI−), and elemental analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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