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Discovery of benzo[g]indol-3-carboxylates as potent inhibitors of microsomal prostaglandin E_2 synthase-1

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ABSTRACT

Selective inhibition of pro-inflammatory prostaglandin (PG)E_2 formation via microsomal PGE_2 synthase-1 (mPGES-1) might be superior over inhibition of all cyclooxygenase (COX)-derived products by non-steroidal anti-inflammatory drugs (NSAIDs) and coxibs. We recently showed that benzo[*g*]indol-3-carboxylates potently suppress leukotriene biosynthesis by inhibiting 5-lipoxygenase. Here, we describe the discovery of benzo[*g*]indol-3-carboxylates as a novel class of potent mPGES-1 inhibitors ($IC_{50} \geqslant 0.1$ μM). Ethyl 2-(3-chlorobenzyl)-5-hydroxy-1*H*-benzo[*g*]indole-3-carboxylate (compound **7a**) inhibits human mPGES-1 in a cell-free assay ($IC_{50} = 0.6$ μM) as well as in intact A549 cells ($IC_{50} = 2$ μM), and suppressed PGE_2 pleural levels in rat carrageenan-induced pleurisy. Inhibition of cellular COX-1/2 activity was significantly less pronounced. Compound **7a** significantly reduced inflammatory reactions in the carrageenan-induced mouse paw edema and rat pleurisy. Together, based on the select and potent inhibition of mPGES-1 and 5-lipoxygenase, benzo[g]indol-3-carboxylates possess potential as novel anti-inflammatory drugs with a valuable pharmacological profile.

1. Introduction

Prostaglandin (PG)E_2 is synthesized from arachidonic acid (AA) by the concerted action of cyclooxygenases (COX) and PGE_2 synthases (PGES). As a key mediator in inflammation, PGE_2 induces fever and pain via specific G-protein coupled receptors EP1-4.[1] Different terminal isoforms of PGES exist, and co-expression studies have indicated preferential functional coupling between COX and PGES isoenzymes.[2] Thus, the cytosolic PGE_2 synthase (cPGES) generally receives its substrate PGH_2 from COX-1 and the microsomal PGE_2 synthase-1 (mPGES-1) from COX-2.[2,3] cPGES is constitutively expressed and primarily provides PGE_2 for homeostasis, whereas expression of mPGES-1 (together with COX-2) is strongly induced by pro-inflammatory stimuli (e.g., interleukin (IL)-1β) resulting in increased PGE_2 formation during inflammation.[4] Results from mPGES-1 inhibitor and knockout studies suggest beneficial effects of targeting mPGES-1 as compared to inhibition of COX-1 and -2 by non-steroidal anti-inflammatory drugs (NSAIDs) or exclusively COX-2 by coxibs.[5] For example, the selective and orally active mPGES-1 inhibitor 2-(6-chloro-1*H*-phenanthro[9,10-*d*]imidazol-2-yl)isophthalonitrile (MF63) is effective in inhibiting LPS-induced pyresis, hyperalgesia, and iodoacetate-induced osteoarthritic pain in guinea pig and in knock-in mice expressing human mPGES-1.[6] Gastrointestinal toxicity, symptomatic for NSAIDs, was observed neither in the knock-in mouse model nor in non-human primates. mPGES-1-deficient mice develop normally but exhibit reduced inflammatory responses[7] and show no fever in response to lipopolysaccharide (LPS).[8] In contrast to coxibs, cardiovascular functions seem not impaired, since mPGES-1 deletion neither affected thrombogenesis nor blood pressure in mice.[9]

The number of mPGES-1 inhibitors described in the literature is limited. Compound **1** (MK-886 (3-[1-(4-chlorobenzyl)-3-*t*-butylthio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid, $IC_{50} = 2$ μM) and structural derivatives thereof potently inhibit cell-free mPGES-1, but they suffer from plasma protein binding and loose potency in cell-based assays or in in vivo animal models.[10,11] On the other hand, phenanthrene imidazoles,[12] α-alkyl-substituted pirinixic acid derivatives,[13] and 2-[6-(4-chlorophenyl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1*H*-pyrrolizin-5-yl] acetic acid (licofelone,[14] currently undergoing phase III trials for therapy of osteoarthritis) inhibit mPGES-1 in cell-free assays and in intact cells or in human whole

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blood. Moreover, diverse natural compounds including the acylphloroglucinols myrtucommulone from *Myrtus communis* and garcinol from *Guttiferae* species or the polyphenolic curcumin from *Curcuma longa* suppressed PGE_2 formation in whole blood, which could be attributed to direct inhibition of mPGES-1.[15–17]

We have recently shown that 5-lipoxygenase (LO) is potently inhibited by diverse indol-3-carboxylates.[18,19] Here, we show that benzo[*g*]indol-3-carboxylates potently inhibit mPGES-1 and thus represent a novel class of dual 5-LO/mPGES-1 inhibitors. Using A549 lung carcinoma cells, we demonstrate that select compounds also suppress PGE_2 formation in intact cells without marked concomitant inhibition of the cellular synthesis of other COX-1/2 products. Finally, significantly reduced PGE_2 levels in pleural exudates of carrageenan-treated rats demonstrate efficacy in vivo after intraperitoneal administration of the benzo[*g*]indol-3-carboxylate **7a**.

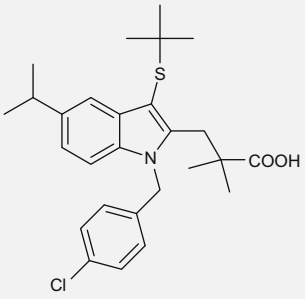
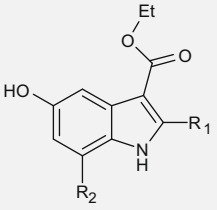
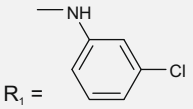
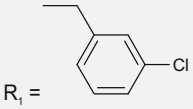
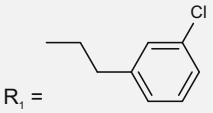
2. Results and discussion

2.1. Screening of indole-3-carboxylates as direct mPGES-1 inhibitors

Upon stimulation with IL-1β (1 ng/ml, 72 h), A549 cells highly express mPGES-1, and after subcellular fractionation, mPGES-1 is found in the microsomal fraction.[4] In order to investigate whether the test compounds inhibit the catalytic activity of mPGES-1, microsomal preparations of IL-1β-stimulated A549 cells (5 μg/100 μl) were pre-incubated with the test compounds for 15 min 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) modified according to Jakobsson et al.[4] Formed PGE_2 was quantified by RP-HPLC. The cyclic peroxide PGH_2 is highly reactive and spontaneously degrades to $PGF_{2\alpha}$, PGD_2, and PGE_2 in aqueous solutions within a few minutes.[20,21] Consequently, the enzymatic assay was performed at 4 °C for 1 min in order to avoid non-enzymatic conversion of PGH_2 to PGE_2. The indole derivative **1**, a well-recognized inhibitor of mPGES-1,[10] was used as reference compound. In accordance with the literature,[11,14,22] compound **1** concentration-dependently (0.1–10 μM) blocked PGE_2 formation with an $IC_{50} \approx 2$ μM showing maximal inhibition (82 ± 3%) at 30 μM.

The test compounds (Table 1) were first analyzed for inhibition of PGE_2 formation in the cell-free assay described above at 10 μM. For compounds that achieved more than 50% inhibition at 10 μM, IC_{50} values were assessed. Compound **2** (ethyl 2-[(3-chlorophenyl)amino]-5-hydroxy-1*H*-indole-3-carboxylate) that showed

Table 1
Inhibition of PGE_2 formation in microsomal preparations of IL-1β-stimulated A549 cells and of isolated ovine COX-1 and human recombinant COX-2

Compound	Structure	mPGES-1 activity (percentage of control) at 10 μM	IC_{50} (μM) mPGES-1	COX activity (percentage of control) at 10 μM	
				COX-1	COX-2
1		23 ± 2***	2[a]	42 ± 7**,[b]	79 ± 8[b]
Indole-3-carboxylates					
2	 R_1 = R_2 = -H	83 ± 8	n.d.[c]	n.d.	n.d.
3	 R_1 = R_2 = -H	55 ± 10***	n.d.	n.d.	n.d.
4	 R_1 = R_2 = -H	65 ± 6***	n.d.	n.d.	n.d.

(*continued on next page*)

Table 1 (*continued*)

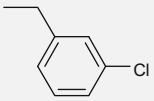
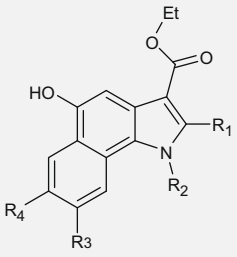
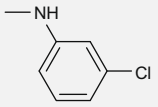
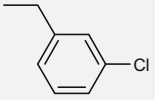
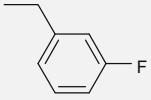
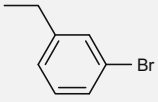
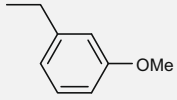
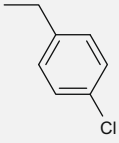
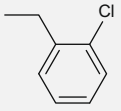
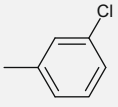
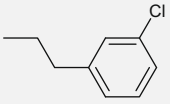
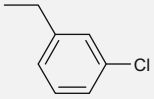
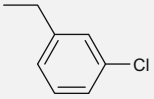
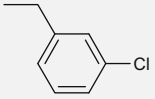
Compound	Structure	mPGES-1 activity (percentage of control) at 10 μM	IC_{50} (μM) mPGES-1	COX activity (percentage of control) at 10 μM	
				COX-1	COX-2
5	 R_1 = R_2 = -4-biphenyl	25 ± 2***	3.1	n.i.[d]	n.i.
Benzo[*g*]-indole-3-carboxylates					
6	 R_1 = R_2, R_3, R_4 = -H	13 ± 4***	1.6	n.i.	57 ± 4***
7a	 R_1 = R_2, R_3, R_4 = -H	3 ± 2***	0.6	12 ± 8***	33 ± 11***
7b	 R_1 = R_2, R_3, R_4 = -H	5 ± 2***	0.5	n.i.	71 ± 3*
7c	 R_1 = R_2, R_3, R_4 = -H	14 ± 5***	0.2	82 ± 6	74 ± 10
7d	 R_1 = R_2, R_3, R_4 = -H	9 ± 1***	0.6	89 ± 10	n.i.
8	 R_1 = R_2, R_3, R_4 = -H	26 ± 7***	3.4	26 ± 10***	47 ± 13***
9	 R_1 = R_2, R_3, R_4 = -H	14 ± 0***	0.1	89 ± 5	66 ± 5
10	 R_1 = R_2, R_3, R_4 = -H	46 ± 6***	9.2	n.d.	n.d.

Table 1 (*continued*)

Compound	Structure	mPGES-1 activity (percentage of control) at 10 μM	IC_{50} (μM) mPGES-1	COX activity (percentage of control) at 10 μM	
				COX-1	COX-2
11	 R_1 = R_2, R_3, R_4 = -H	57 ± 6***	n.d.	n.d.	n.d.
12	 R_1 = R_2 = -benzyl R_3, R_4 = -H	n.i.	n.d.	n.d.	n.d.
13	 R_1 = R_2 = -H R_3, R_4 = -OMe	30 ± 2***	1.7	n.i.	n.i.
14	 R_1 = R_2, R_4 = -H R_3 = -phenyl	28 ± 5***	2.8	66 ± 8	n.i.

Mean values (n = 3–5) and standard error estimates are given; *p <0.05; **p <0.01; ***p <0.001.

[a] Ref. 14.

[b] Published by Koeberle et al.[41]

[c] n.d. = not determined.

[d] n.i. = no inhibition.

potent inhibition of 5-LO in cell-based (IC_{50} = 2.4 μM) and cell-free assays (IC_{50} = 0.3 μM)[18] was used as lead structure (Table 1). Only a moderate inhibition of mPGES-1 activity was observed at 10 μM (17 ± 8%). Exchange of the nitrogen by a methylene moiety (**3**) and elongation by another methylene group (**4**) slightly improved the potency. Removal of the 5-hydroxy moiety in **2** or replacement by chlorine had no marked impact on the inhibition of mPGES-1 activity (data not shown). It has been shown that bulky hydrophobic substituents (i.e., biphenyl moieties) in mPGES-1 inhibitors like in derivatives of **1**[11] or in pirinixic acid derivatives[13] strongly enhance the potency. These substituents may bind to a large hydrophobic pocket formed by Val37, Val128, and Thr131 in the enzyme.[23] In fact, when a 4-biphenyl residue at 7-position of **2** was introduced (**5**), the potency was significantly increased (IC_{50} = 3.1 μM). Enlargement of the hydrophobic core structure by annelation of benzene to the indole of **2**, yielding the corresponding benzo[*g*]indole derivate **6**, even led to an IC_{50} of 1.6 μM. Such benzo[*g*]indole-3-carboxylates were also found to be potent inhibitors of 5-LO being superior over the corresponding compounds lacking the annelated benzene.[19] Therefore, we further explored benzo[*g*]indole-3-carboxylates in order to obtain potent mPGES-1 inhibitors.

The exchange of the nitrogen in 2-position of **6** by a methylene group, leading to **7a**, further enhanced the potency with essentially complete suppression of PGE_2 formation at 10 μM (97 ± 2% inhibition), and the IC_{50} value was determined at 0.6 μM. Replacement of chlorine in **7a** by fluorine (compound **7b**), bromine (compound **7c**), or a methoxy group (**7d**) was tolerated and yielded somewhat lower IC_{50} values, although the effectiveness at 10 μM was not improved over **7a**. Variation of the positioning of the chlorine in the benzyl ring of **7a** to *para* (compound **8**) was detrimental, whereas *ortho*-positioning (compound **9**) led to an IC_{50} = 0.1 μM and strong inhibition at 10 μM (86 ± 0.4%). When the distance between the phenyl ring in 2-position and the indole in **7a** was altered, either by direct connection of the 3-chlorophenyl residue (**10**) or elongation by insertion of another methylene moiety (**11**), the potency was markedly lost, and the IC_{50} values shifted to approx. 9 μM and >10 μM, respectively. A total loss of activity was evident, when the indole nitrogen of **7a** was substituted by a benzyl group (**12**), and also the corresponding benzyl carboxylate of **7a** was clearly inferior (42 ± 8% inhibition at 10 μM, not shown). Thus, the heterocyclic nitrogen and the carboxylate group were excluded from further structural optimization. Further enlargement of the hydrophobic core structure of **7a** by annelation of a 2,3-dimethoxybenzene moiety (**13**) to the indole, instead of benzene, or phenyl substitution at 8-position of the benzo[*g*]indole (**14**) also failed to improve the efficiency and led to compounds with higher IC_{50} values. Together, we identified 2-benzyl-substituted ethyl 5-hydroxy-benzo[*g*]indole-3-carboxylates as direct and potent inhibitors of mPGES-1.

2.2. Analysis of the inhibition of COX isoenzymes by indole-3-carboxylates

Using purified ovine COX-1 and purified human recombinant COX-2, we determined the effects of the test compounds on isolated COX isoenzymes. COX-1/2 was pre-incubated with the test compounds at 10 μM for 5 min, AA (5 μM for COX-1, 2 μM for

COX-2) was added, and after 5 min at 37 °C, the formation of 12(*S*)-hydroxy-5-*cis*-8,10-*trans*-heptadecatrienoic acid (12-HHT), the major COX-1/2-derived product under these experimental conditions,[14] was determined. Indomethacin (10 μM) and celecoxib (5 μM), used as controls, inhibited 12-HHT formation by 95±0.2% and 70±11%, respectively. The benzo[*g*]indole-carboxylate **7a** significantly suppressed both COX-1 and -2 activity. Inhibition of COX isoenzymes was strongly impaired by replacement of chlorine in **7a** by fluoro (**7b**), bromo (**7c**) or methoxy substituents (**7d**) and by shifting the *meta*-chloro substituent to *para* (**8**) or *ortho* (**9**) position. Also the 7-biphenyl-substituted indole-3-carboxylate **5** and the 7- and/or 8-substituted benzo[*g*]indole-3-carboxlates **13** and **14** failed to significantly affect COX-1/2 activity.

2.3. Characterization of mPGES-1 inhibition by benzo[*g*]indole-3-carboxylates

To investigate whether the benzo[*g*]indole-3-carboxylates inhibit mPGES-1 in a reversible or irreversible manner, we performed wash-out experiments. To this aim, 10-fold concentrated microsomal preparations of A549 cells (50 μg/100 μl) were pre-incubated with 3 μM compound **1** (a reversible inhibitor[14] used as control) or **7a**. After 15 min, these samples were diluted 10 times to obtain an inhibitor concentration of 0.3 μM (labeled '3(0.3)') and 50 μg/1000 μl of microsomes, and to an aliquot of 100 μl thereof, PGH_2 was added in order to initiated PGE_2 formation. For comparison, 5 μg/100 μl of microsomal preparations were directly pre-incubated with 0.3 μM (labeled '0.3') or 3 μM (labeled '3') compounds (no dilution).

Both compounds **1** and **7a** only marginally reduced PGE_2 synthesis at 0.3 μM, whereas a potent mPGES-1 inhibition was evident at 3 μM (Fig. 1A). Upon 10-fold dilution of the samples containing 3 μM of **7a** or of **1**, the suppressed PGE_2 synthesis was restored, implying a reversible mode of inhibition.

The potency of compound **1** to inhibit mPGES-1 is independent of the substrate concentration (i.e., 1–20 μM PGH_2), implying a non-competitive mode of action.[14] Similarly, inhibition of mPGES-1 activity by **7a** in the cell-free assay was not different at high (20 μM PGH_2) or low (1 μM PGH_2) substrate concentration, concluding that also benzo[*g*]indole-3-carboxylates may act at physiological concentrations of PGH_2 in a substrate concentration-independent manner (Fig. 1B).

2.4. Benzo[*g*]indole-3-carboxylates selectively inhibit PGE_2 formation in cell-based assays

Potent inhibition of mPGES-1 and/or COX-1/2 under cell-free conditions must not necessarily result in efficient suppression of cellular prostanoid formation. In fact, compound **1** and related derivatives with high potency on isolated mPGES-1 failed to efficiently inhibit PGE_2 biosynthesis in intact A549 cells or in human whole blood.[11] IL-1β-stimulated A549 cells highly express COX-2 and mPGES-1[4], whereas COX-1 cannot be detected.[24] Accordingly, PGE_2 formation in A549 cells depends on the concerted action of the functionally coupled enzymes COX-2 and mPGES-1. Nevertheless, other PGE_2 synthases, namely mPGES-2 and cPGES (essentially coupled to COX-1) may still participate to a minor extent. After pre-incubation of intact IL-1β-stimulated A549 cells with either **6**, **7a**, or **9**, cells were activated by 2.5 μM Ca^{2+} ionophore A23187. Exogenous AA (1 μM AA and 18.4 kBq [^{3}H]AA) was provided to circumvent the need for release of endogenous AA and thus to exclude possible effects on AA-releasing PLA_2 enzymes. The COX-2 selective celecoxib and compound **1** were used as controls. In agreement with previous studies,[14] PGE_2 formation was almost completely abolished by 5 μM celecoxib (not shown), whereas 37 ± 6% PGE_2 was still produced in the presence of 30 μM compound **1**. As shown in Figure 2A, compounds **6** and **7a** concentration-dependently suppressed PGE_2 formation with an IC_{50} of 7.9 and 2.0 μM, respectively. The *ortho*-chlorobenzyl-substituted **9** was less potent with an IC_{50} of 20.5 μM in intact A549 cells (Fig. 2A), although it displayed the lowest IC_{50} value (i.e., 0.1 μM) of all tested compounds in the cell-free assay. Similarly, compound **9** was less potent to inhibit 5-LO in intact neutrophils (IC_{50} = 1.2 μM) versus cell-free assays (IC_{50} = 0.097 μM), while the difference between the corresponding values for compound **7a** was less pronounced (IC_{50} = 0.23 and 0.086 μM in neutrophils and cell-free assay, respectively).[19] As observed before with other mPGES-1 inhibitors, PGE_2 formation was not completely blocked by the benzo[*g*]indole-3-carboxylates, and a residual PGE_2 formation of approx. 35–40% still remained. Since **7a** already reduced PGE_2 synthesis by 65 ± 8% at 3 μM without further inhibition at 10 or 30 μM, the remaining PGE_2 formation is presumably mPGES-1-independent and does not originate from an incomplete inhibition of mPGES-1 activity.

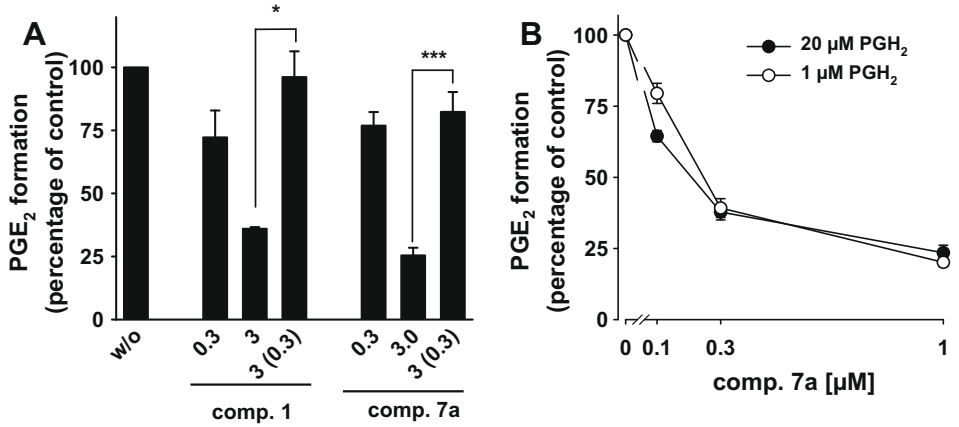


Figure 1. The benzo[*g*]indol-3-carboxylate **7a** inhibits mPGES-1 in a reversible and substrate concentration-independent manner. (**A**) Microsomal preparations of IL-1β-stimulated A549 cells (50 μg/100 μl) were pre-incubated with 3 μM compound **1** or **7a** for 15 min at 4 °C. The sample was then diluted 10-fold to obtain an inhibitor concentration of 0.3 μM (labeled '3(0.3)') and 50 μg/1000 μl of microsomes, and to an aliquot of 100 μl thereof, PGH_2 (20 μM) was added to initiate PGE_2 formation. For comparison, we directly pre-incubated microsomal preparations (5 μg/100 μl) for 15 min with 0.3 μM (labeled '0.3') or 3 μM (labeled '3') compound **1** or **7a** or with vehicle (DMSO), and then, 20 μM PGH_2 was added (no dilution). Then, PGE_2 formation was analyzed as described in the text. Data are given as mean ± SE, n = 3–4, $^*p$ <0.05 versus vehicle (0.1% DMSO) control, ANOVA + Tukey HSD *post-hoc* tests. (**B**) The potency of **7a** for mPGES-1 inhibition was compared at 1 and 20 μM PGH_2 as substrate. For 1 μM PGH_2, the amount of PGE_2 was below the detection limit of the HPLC and was thus quantified by use of a PGE_2 High Sensitivity EIA Kit according to manufacturer's protocol. Data are given as mean ± SE, n = 3–4.

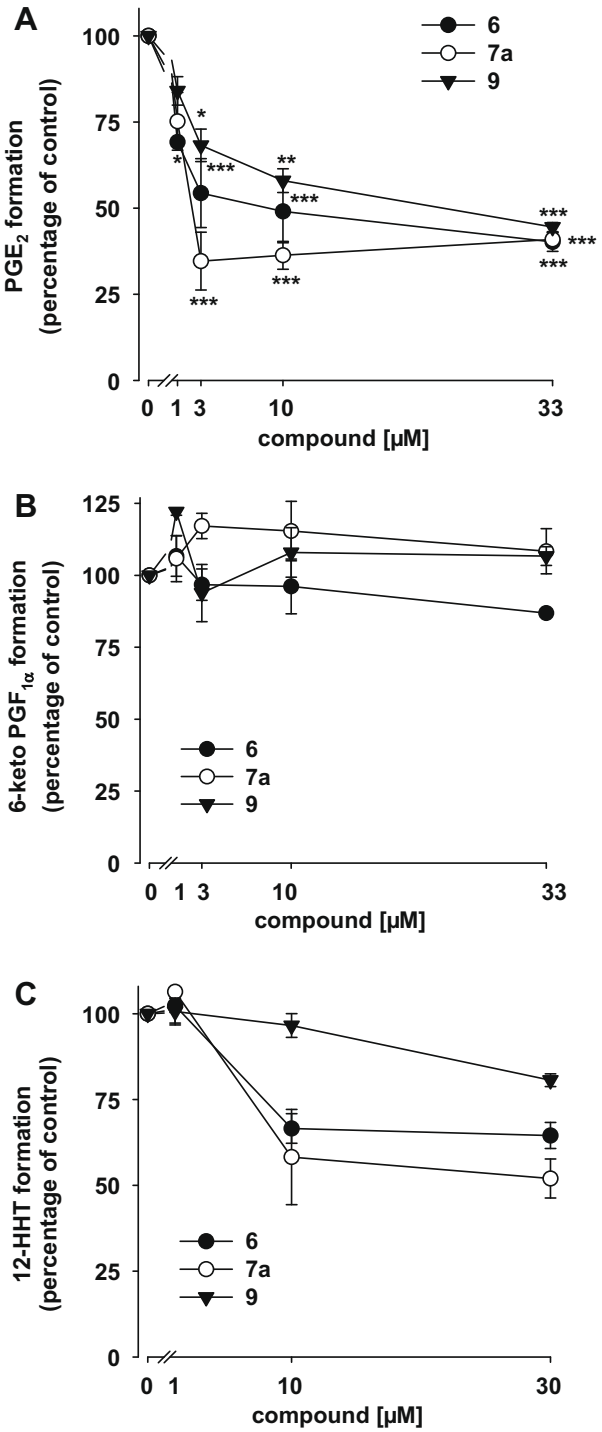


Figure 2. Effects of benzo[*g*]indole-3-carboxylates on the formation of prostanoids in intact cells. (**A**) PGE_2 formation. IL-1β-stimulated A549 cells were pre-incubated with test compounds or vehicle (DMSO) for 10 min, then, cellular PGE_2 synthesis was elicited by addition of 2.5 μM A23187 plus 1 μM AA and [^{3}H]AA (18.4 kBq). After 15 min, formed [^{3}H]PGE_2 was analyzed by RP-HPLC and liquid scintillation counting as described in Section 4. (**B**) 6-Keto $PGF_{1\alpha}$ formation. IL-1β-stimulated A549 cells (10^6/ml) were pre-incubated with the indicated test compounds or vehicle (DMSO) for 15 min prior to addition of 30 μM AA. After 15 min at 37 °C, the amount of 6-keto $PGF_{1\alpha}$ was assessed by ELISA as described in Section 4. Celecoxib (5 μM) was used as control (70 ± 11% inhibition). (**C**) 12-HHT formation. Freshly isolated human platelets (10^8/ml) were pre-incubated with the test compounds or vehicle for 5 min prior to stimulation with AA (5 μM). After 5 min at 37 °C, the formation of 12-HHT was determined by RP-HPLC as described in Section 4. Indomethacin (10 μM) was used as control (95 ± 0.2% inhibition). Data are given as mean ± SE, $n = 3–5$, $^{**}p < 0.01$ or $^{***}p < 0.001$ versus vehicle (0.1% DMSO) control, ANOVA + Tukey HSD *post-hoc* tests.

Side effects of NSAIDs and coxibs mainly depend on an imbalance of COX-derived prostanoids such as PGI_2, PGD_2, and TxA_2 that possess important protective physiological functions in the cardiovascular system and in immunoregulation.[1] Notably, certain benzo[*g*]indol-3-carboxylates (e.g., compound **7a**) possess an inhibitory potential on COX enzymes in cell-free assays. To evaluate whether they can affect cellular prostanoid formation at the level of COX enzymes, we analyzed in parallel the formation of 6-keto $PGF_{1\alpha}$, the stable metabolite of PGI_2, by ELISA as a biological parameter of COX-2 activity. In fact, formation of 6-keto $PGF_{1\alpha}$ was not significantly suppressed by **6** or **9** and also not by **7a**, up to 30 μM (Fig. 2B). Celecoxib (5 μM) efficiently reduced 6-keto $PGF_{1\alpha}$ formation by 70 ± 11% under these conditions.

For analysis of the effects of benzo[*g*]indole-3-carboxylates on cellular COX-1 activity, freshly isolated human platelets (supplemented with 5 μM exogenous AA) were used. Platelets are a rich source of COX-1, and are generally applied in cell-based models to assess inhibition of COX-1 in a biological environment.[25] Platelets were pre-incubated with the test compounds prior to addition of 5 μM AA, and 12-HHT, the major COX-1 product formed under these conditions, was analyzed by RP-HPLC. Indomethacin (10 μM) was used as control and potently inhibited 12-HHT formation (95 ± 0.2%). In contrast, **6** and **7a** moderately suppressed 12-HHT formation at 10 μM (by 31 ± 3% and 43 ± 11%, respectively), and no significant effect was observed for **9** up to 30 μM (Fig. 2C). Together, one may conclude that the observed inhibition of cellular PGE_2 by the benzo[*g*]indole-3-carboxylates cannot be attributed to an interference with PGH_2 formation, and thus, COX-1 and -2 as possible targets in intact cells are unlikely.

2.5. Anti-inflammatory effectiveness of the benzo[*g*]indole-3-carboxylate 7a, and inhibition of PGE_2 biosynthesis in vivo

Because **7a** was most efficient to inhibit PGE_2 formation in intact A549 cells among the benzo[*g*]indol-3-carboxylates tested (see Fig. 2A) and also potently suppressed leukotriene (LT)B_4 formation in cell-based models,[19] its anti-inflammatory effectiveness in vivo was evaluated in two well-established animal models of inflammation, the carrageenan-induced mouse paw edema and the carrageenan-induced pleurisy in rats. During paw edema formation, PGE_2 levels are significantly elevated,[26,27] and COX-inhibitors were shown to prevent the inflammatory response.[28] Also in the pleurisy model, PGE_2 essentially contributes to the early inflammatory response following carrageenan stimulation.[29,30]

For analysis of the effects of **7a** on mouse paw edema, the compound (0.25, 1, and 4 mg/kg) was administered intraperitoneally (ip) 30 min prior to injection of carrageenan into the mouse paw. The increase in paw volume (vehicle ip treatment) reached a maximum at 4 h post-carrageenan treatment (Fig. 3). In fact, in mice treated with 0.25, 1, and 4 mg/kg **7a**, the peak of the response to carrageenan at 4 h was reduced by 37%, 53%, and 61%, respectively. Indomethacin (5 mg/kg, ip), used as reference, caused 57% inhibition (Fig. 3).

In the rat pleurisy model, ip treatment of rats with 4 mg/kg of **7a**, 30 min prior to carrageenan administration, reduced the inflammatory reaction (measured as exudate volume and inflammatory cell numbers in the pleural exudates) as described before[19] (Table 2). Indomethacin (5 mg/kg, ip) reduced exudate formation to a similar degree as **7a** but was somewhat more efficient in reducing cell infiltration. In agreement with the results from intact A549 cells, **7a** significantly lowered the pleural PGE_2 levels but had only a small and non-significant effect on 6-keto $PGF_{1\alpha}$ levels, excluding a general interference with the biosynthesis of all PGs or an effect at the level of COX enzymes. Indomethacin was considerably more efficient in lowering PGE_2 levels but also blocked formation of 6-keto $PGF_{1\alpha}$ (Table 2). As reported before, **7a** reduced

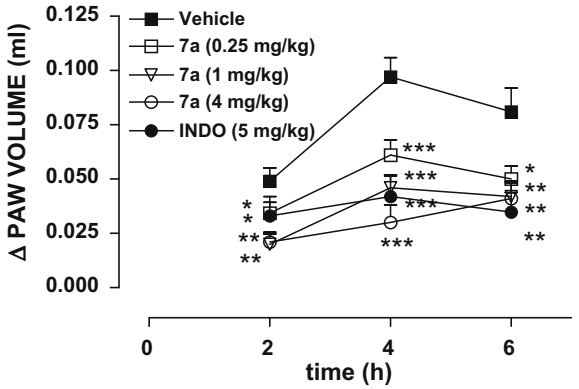


Figure 3. Effects of the benzo[*g*]indole-3-carboxylate **7a** on carrageenan-induced mouse paw edema. Mice ($n = 10$ for each experimental group) were treated ip with 0.25, 1, and 4 mg/kg **7a**, 5 mg/kg indomethacin (indo), or vehicle (2% DMSO) 30 min before carrageenan subplantar injection. Data are given as mean ± SE, $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$ versus vehicle (DMSO 2%) control, ANOVA + Tukey HSD *post-hoc* tests.

LTB_4 levels in the pleural exudates,[19] and we conclude that both suppression of LTB_4 and PGE_2 may contribute to the anti-inflammatory effect. Indomethacin, in contrast, did not suppress LTB_4 levels (Table 2), which precludes that general anti-inflammatory effects (due to suppression of PGE_2 formation by **7a**) are responsible for the reduction of LTB_4 levels. In conclusion, our data support the anti-inflammatory effectiveness of **7a** in living animals and provide evidence for a selective suppression of PGE_2 in vivo without significantly affecting the biosynthesis of PGI_2.

The current therapeutic application of NSAIDs such as aspirin or indomethacin suffers from gastrointestinal and renal side effects due to the overall suppression of COX-derived prostanoid biosynthesis. COX-2 selective coxibs have been designed lacking gastrointestinal toxicity, though they increase the cardiovascular risk.[1] Knockout experiments and in vivo studies suggest that selective inhibitors for mPGES-1 may achieve the therapeutic efficiency of NSAIDs while circumventing their adverse events at the same time.[31] Moreover, dual inhibition of the PG and the LT synthetic pathway might be of advantage in terms of higher anti-inflammatory efficacy on one hand and in terms of lower side effects on the other.[32] In particular, dual PG/LT synthetic pathway inhibitors almost completely diminished gastric toxicity.[33] Although the underlying mechanism is not completely understood, inhibition of LT formation in addition to repressing PGE_2 seemingly possesses advantages compared to single interference. For example, licofelone blocks the biosynthesis of both PGE_2 and LTs[34] by inhibiting mPGES-1[14] and the 5-LO-activating protein (FLAP),[35] respectively, and showed potent anti-inflammatory efficacy in clinical trials and animal studies with low gastric side effects[36] and even beneficial cardiovascular properties.[37] Unfortunately, mice deficient in both mPGES-1 and 5-LO (or FLAP) have not been created yet, but such animals might certainly allow additional insights into the suitability of the 'dual inhibition concept'.

3. Conclusion

In summary, we have reported the discovery of benzo[*g*]indole-3-carboxylates as novel class of mPGES-1 inhibitors. Members of this structural class are able to completely inhibit mPGES-1 activity in cell-free assays at 10 μM (**7a**) and exhibit IC_{50} values of 0.1 μM (**9**). We found that *ortho*-, *meta*-, or *para*-halogenated benzyl-substituents in 2-position of the indole led to a strong potency towards mPGES-1. In contrast to the SARs regarding 5-LO, either shortening or elongation of the distance between the indole and the halogenated aryl moiety clearly diminished the potency. Also, benzyl substitution of the indole nitrogen or benzyl esterification instead of ethyl was clearly detrimental. Selected compounds (i.e., **6**, **7a**, and **9**) from the benzo[*g*]indole-3-carboxylate series showed marked inhibition of PGE_2 formation in intact (A549) cells (**7a**, $IC_{50} = 2$ μM), and compound **7a** also reduced PGE_2 levels in vivo, accompanied by high anti-inflammatory efficacy. Although **7a** inhibited the activities of isolated COX-1/2, inhibition of cellular COX-1/2 product formation was considerably less pronounced, suggesting a selective interference with mPGES-1. It is remarkable that the benzo[*g*]indole-3-carboxylate **7a** combines strong inhibition of mPGES-1 with potent suppression of cellular 5-LO product formation ($IC_{50} = 0.23$ μM) by direct inhibition of 5-LO ($IC_{50} = 0.086$ μM).[19] Such a dual inhibition may be valuable regarding the anti-inflammatory efficacy but also in terms of lower gastric toxicity versus NSAIDs. Moreover, the failure of **7a** to abolish PGI_2 biosynthesis, reflected by the failure to repress 6-keto $PGF_{1\alpha}$ formation in intact A549 cells or in the pleurisy model, proposes a lower cardiovascular risk as compared to selective COX-2 inhibitors. The numerous encouraging results obtained in animal studies and clinical trials with the dual PG and LT synthesis inhibitor licofelone[38] are tempting to deduce similar or even improved properties for select benzo[*g*]indole-3-carboxylates. Future studies addressing their anti-inflammatory effectiveness in other species and additional models but also evaluation of the gastric toxicity and cardiovascular risk as well as general toxicity will reveal the therapeutic potential of (these drug-like) benzo[*g*]indole-3-carboxylates.

4. Experimental

4.1. Compounds

Compounds **2–14** were synthesized as previously reported.[18,19] All structures were confirmed by ^{1}H and ^{13}C NMR, infrared spectroscopy as well as by mass spectrometry (ESI-), and the purity (>98%) was checked by combustion analysis as described.[19]

4.2. Assay systems

4.2.1. Materials

The anti-6-keto $PGF_{1\alpha}$ antibody was a generous gift by Dr. T. Dingermann (University of Frankfurt, Germany). Materials used: DMEM/high glucose (4.5 g/l) medium, penicillin, streptomycin,

Table 2
Effect of **7a** on carrageenan-induced pleurisy in rats and pleural prostanoid levels

Treatment	Exudate volume (ml)	Inflammatory cells × 10^6	PGE_2 (ng/rat)	6-Keto $PGF_{1\alpha}$ (ng/rat)	LTB_4 (ng/rat)
Vehicle	0.48 ± 0.08[a]	46.67 ± 3.53[a]	2.79 ± 0.39	3.00 ± 1.02	1.17 ± 0.21[a]
Compd **7a** (4 mg/kg)	0.11 ± 0.0026***,[a]	28 ± 6.83*,[a]	1.30 ± 0.19**	2.02 ± 0.64	0.60 ± 0.096*,[a]
Indomethacin (5 mg/kg)	0.14 ± 0.027***,[a]	18 ± 2.5***,[a]	0.33 ± 0.04***	0.018 ± 0.007**	1.02 ± 0.19

Thirty minutes before intrapleural injection of carrageenan, rats ($n = 10$ for each experimental group) were treated ip with 4 mg/kg **7a**, 5 mg/kg indomethacin, or vehicle (DMSO 4%). Exudate volume, PGE_2, 6-keto $PGF_{1\alpha}$, and LTB_4 levels as well as inflammatory cell accumulation in the pleural cavity were assessed 4 h after carrageenan injection. Mean values (n = 3-5) and standard error estimates are given; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.01$ versus vehicle.

[a] Published by Karg et al.[19]

trypsin/EDTA solution, PGH_2, Larodan (Malmö, Sweden); 11β-PGE_2, PGB_1, MK-886, 6-keto $PGF_{1\alpha}$ and LTB_4 enzyme immunoassays, Cayman Chemical (Ann Arbor, MI); [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). For animal studies, **7a** and indomethacin (1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indole-3-acetic acid) were dissolved in DMSO and diluted with saline achieving a final DMSO concentration of 2–4%. [^{3}H]-PGE_2 was from Perkin–Elmer Life Sciences (Milan, Italy) and PGE_2 antibody from Sigma–Aldrich (Milan, Italy). All other chemicals were obtained from Sigma–Aldrich (Deisenhofen, Germany) unless stated otherwise.

4.2.2. Cells and cell viability assay

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 (30 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, Perkin–Elmer, Rodgau-Juegesheim, Germany). Neither **6** nor **7a** significantly reduced cell viability (data not shown), excluding possible acute cytotoxic effects of the compounds in the cellular assays.

4.2.3. Induction of mPGES-1 expression in A549 cells and isolation of microsomes

Preparation of A549 cells and determination of mPGES-1 activity was performed as described previously.[14] 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 FCS (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 homogenization 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.

4.2.4. Determination of PGE_2 synthase activity in microsomes of A549 cells

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 using acetonitrile (200 μl) as eluent, and analyzed by RP-HPLC (30% acetonitrile aq + 0.007% TFA (v/v), Nova-Pak® C18 column, 5 × 100 mm, 4 μm particle size, flow rate 1 ml/min) with UV detection at 195 nm as previously described.[14] 11β-PGE_2 was used as internal standard to quantify PGE_2 product formation by integration of the area under the peaks. IC_{50} values were assessed by five determinations at compound concentrations of 0.01, 0.03, 0.1, 0.3, 1, 3, 10, and 30 μM.

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

Inhibition of the activities of isolated COX-1 and -2 was performed as described.[14] 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 hemoglobin, 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-HHT was extracted and then analyzed by HPLC.

4.2.6. Determination of PGE_2 and 6-keto $PGF_{1\alpha}$ formation in intact A549 cells

IL-1β-treated A549 cells were cultured as described above. 4×10^6 cells per ml PBS containing $CaCl_2$ (1 mM) were pre-incubated with test compounds or vehicle (DMSO, never exceeding a final concentration of 0.3%) at 37 °C for 10 min, and PGE_2 formation was started by addition of ionophore A23187 (5-methylamino-2-[[(2*S*,3*R*,5*R*,8*S*,9*S*)-3,5,9-trimethyl-2-[(2*S*)-1-oxo-1-(1*H*-pyrrol-2-yl)-propan-2-yl]-1,7-dioxaspiro[5.5]undecan-8-yl]methyl]-1,3-benzoxazole-4-carboxylic acid, 2.5 μM), AA (1 μM), and [^{3}H]AA (18.4 kBq). The reaction was stopped after 15 min on ice. After centrifugation (800*g*, 5 min, 4 °C), the supernatant was acidified (pH 3) by addition of citric acid (20 μl, 2 M), and the internal standard 11β-PGE_2 (2 nmol) was added. Radiolabeled PGE_2 was separated by RP-18 solid phase extraction and HPLC analysis 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.

For determination of 6-keto $PGF_{1\alpha}$, 5×10^6 cells, resuspended in 1 ml PBS containing $CaCl_2$ (1 mM), were pre-incubated with the indicated compounds or celecoxib (4-(5-(4-methylphenyl)-3-(trifluoromethyl)pyrazol-1-yl)benzenesulfonamide, 5 μM) for 15 min at 37 °C, and 6-keto $PGF_{1\alpha}$ formation was initiated by addition of AA (30 μM). After 15 min at 37 °C, the reaction was stopped on ice. Cells were centrifuged (300*g*, 5 min, 4 °C), and the amount of released 6-keto $PGF_{1\alpha}$ was assessed by ELISA using a monoclonal antibody against 6-keto $PGF_{1\alpha}$ according to the protocol described by Yamamoto et al.[39] For the ELISA, the monoclonal antibody (0.2 μg/200 μl) was coated on microtiter plates via a goat anti-mouse immunoglobulin G antibody. 6-Keto $PGF_{1\alpha}$ (15 μg) was linked to bacterial β-galactosidase (0.5 mg), and the enzyme activity bound to the antibody was determined in an ELISA reader at 550 nm (reference wavelength: 630 nm) using chlorophenol-red-β-D-galactopyranoside (CPRG, Roche Diagnostic GmbH) as substrate.

4.2.7. Determination of COX-1 product formation in washed platelets

Freshly isolated platelets (10^8/ml PBS) were supplemented with 1 mM $CaCl_2$ and pre-incubated with the indicated agents (final DMSO concentration $\leqslant$ 0.3%) for 10 min at room temperature. After addition of AA (5 μM) and further incubation for 5 min at 37 °C, the

COX-1 product 12-HHT was extracted and then analyzed by HPLC as described.[40]

4.2.8. Carrageenan-induced pleurisy in rats and paw edema in mice

For analysis of pleurisy, test compounds were given ip to male Wistar Han rats (220–230 g) 30 min before carrageenan. Rats were anaesthetized and λ-carrageenan was injected into the pleural cavity. After four hours, the exudate was removed, and the amount was measured. Infiltrated leukocytes in the exudate were counted by light microscopy after vital trypan blue staining. The amount of PGE_2 and 6-keto $PGF_{1\alpha}$ in the exudate was assayed by radioimmunoassay and enzyme immunoassay, respectively. The results are expressed as nanograms per rat and represent the mean ± SE of 10 rats.

For analysis of paw edema, test compounds were given ip to male adult CD1 mice 30 min before carrageenan. Each group of animals received subplantar administration of saline (0.05 ml) or λ-carrageenan 1% type IV (w/v) (0.05 ml) in saline. The paw was immerged in the measurement chamber and the volume was measured by using a special hydropletismometer immediately before subplantar injection and 2, 4, and 6 h thereafter. The assessment of paw volume was always performed in double blind. More methodological details can be found in the Supplementary data.

4.2.9. Statistics

Data are expressed as mean ± SE. IC_{50} values were graphically calculated from averaged measurements at five different concentrations of the compounds (0.01–33 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.

Acknowledgments

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A. Supplementary data

Supplementary data (the detailed description of the performance and analysis of carrageenan-induced pleurisy in rats and paw edema in mice) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.10.025.

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