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RESEARCH PAPER

Molecular pharmacological profile of a novel thiazolinone-based direct and selective 5-lipoxygenase inhibitor

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BACKGROUND AND PURPOSE

The potency of many 5-lipoxygenase (5-LOX) inhibitors depends on the cellular peroxide tone and the mechanism of 5-LOX enzyme activation. Therefore, new inhibitors that act regardless of the mode of enzyme activation need to be developed. Recently, we identified a novel class of thiazolinone-based compounds as potent 5-LOX inhibitors. Here, we present the molecular pharmacological profile of (*Z*)-5-(4-methoxybenzylidene)-2-(*p*-tolyl)-*5H*-thiazol-4-one, compound C06.

EXPERIMENTAL APPROACH

Inhibition of 5-LOX product formation was determined in intact cells [polymorphonuclear leukocytes (PMNL), rat basophilic leukaemia-1, RAW264.7] and in cell-free assays [homogenates, 100 000× *g* supernatant (S100), partially purified 5-LOX] applying different stimuli for 5-LOX activation. Inhibition of peroxisome proliferator-activated receptor (PPAR), cytosolic phospholipase A_2 ($cPLA_2$), 12-LOX, 15-LOX-1 and 15-LOX-2 as well as cyclooxygenase-2 (COX-2) were measured *in vitro*.

KEY RESULTS

C06 induced non-cytotoxic, direct 5-LOX inhibition with IC_{50} values about 0.66 μM (intact PMNL, PMNL homogenates) and approximately 0.3 μM (cell-free PMNL S100, partially purified 5-LOX). Action of C06 was independent of the stimulus used for 5-LOX activation and cellular redox tone and was selective for 5-LOX compared with other arachidonic acid binding proteins (PPAR, $cPLA_2$, 12-LOX, 15-LOX-1, 15-LOX-2, COX-2). Experimental results suggest an allosteric binding distinct from the active site and the C2-like domain of 5-LOX.

CONCLUSIONS AND IMPLICATIONS

C06 was identified as a potent selective direct 5-LOX inhibitor exhibiting a novel and unique mode of action, different from other established 5-LOX inhibitors. This thiazolinone may possess potential for intervention with inflammatory and allergic diseases and certain types of cancer.

Abbreviations

5-LOX, 5-lipoxygenase; AA, arachidonic acid; CML, chronic myeloid leukaemia; $cPLA_2$, cytosolic phospholipase A_2; FLAP, 5-LOX-activating protein; LT, leukotriene; FCS, fetal calf serum; PAPC, 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; PGC buffer, PBS containing 1 $\mathrm{mg \cdot mL^{-1}}$ glucose and 1 mM $CaCl_2$; PMNL, polymorphonuclear leukocytes; RBL, rat basophilic leukaemia; S100, 100 000× *g* supernatant

Introduction

5-Lipoxygenase (5-LOX), a non-haem iron containing dioxygenase, catalyses the biosynthesis of leukotrienes (LTs), which are lipid mediators of inflammatory and allergic responses (nomenclature follows Alexander *et al.*, 2011). LTs exert their biological effects via specific G-protein-coupled receptors and play a pivotal role in inflammatory and allergic disorders as well as in cardiovascular diseases and cancer (Werz and Steinhilber, 2006; Peters-Golden and Henderson, 2007). 5-LOX catalyses the first steps in the conversion of arachidonic acid (AA) into LTA_4, with the aid of the 5-LOX-activating protein (FLAP) (Rådmark *et al.*, 2007). LTA_4 is further converted by LTA_4-hydrolase into LTB_4. Alternatively, conjugation of LTA_4 with glutathione by LTC_4 synthases yields the cysteinyl-containing LTs C_4, D_4 and E_4 that cause bronchoconstriction and vascular permeability (Peters-Golden and Henderson, 2007). As 5-LOX is a key enzyme in biosynthesis of LTs, 5-LOX inhibitors are predicted to be of therapeutic value for the treatment of asthma, allergic rhinitis, atherosclerosis and cancer (Werz and Steinhilber, 2006; Rådmark and Samuelsson, 2010). Moreover, novel indications have emerged in, for example, malaria (Shumilina *et al.*, 2006; Lang *et al.*, 2009), cancer chemoprevention (Menna *et al.*, 2010) or chronic myeloid leukaemia (CML) (Chen *et al.*, 2009). Recently, it was found that the ALOX5-gene is a critical regulator for leukaemia stem cells in BCR-ABL-induced CML (Chen *et al.*, 2009) as the use of the 5-LOX inhibitor zileuton impaired CML. So far, zileuton is the only 5-LOX inhibitor that reached the market in the USA for the treatment of asthma.

Zileuton belongs to the iron ligand-type 5-LOX inhibitors, which chelate the active site iron. There are also redox-type 5-LOX inhibitors that interfere with the catalytic cycle via iron-reducing properties and as a third class of inhibitor, the non-redox-type inhibitors without any reducing properties (Ford-Hutchinson *et al.*, 1994). We and others have shown that several pathways are involved in cellular 5-LOX activation (Werz *et al.*, 2002a,b; Rådmark *et al.*, 2007; Flamand *et al.*, 2009). The enzyme can be activated by an increase in intracellular Ca^{2+} concentration or phosphorylation of 5-LOX via p38-MAPK and ERK. Nuclear membrane association, including co-localization with FLAP, is also an important determinant for 5-LOX activation (Woods *et al.*, 1993; Mandal *et al.*, 2008). The inhibitory potency of many 5-LOX inhibitors depends on the cellular peroxide tone and the mechanism of 5-LOX activation (Werz *et al.*, 1998; Fischer *et al.*, 2003). Thus, many non-redox-type 5-LOX inhibitors show impaired 5-LOX inhibition when peroxide concentration is high or when the enzyme is activated by phosphorylation. Therefore, inhibitors with different pharmacological profiles potently inhibiting the enzyme, regardless of the mode of enzyme activation, are needed and potential drug candidates should be screened in different cellular assays. Moreover, the identification of selective inhibitors within the AA cascade will help to study the role of different AA metabolizing enzymes in the pathology of various diseases.

In the recently published crystal structure of 5-LOX (Gilbert *et al.*, 2011), the enzyme is composed of two domains, the N-terminal regulatory C2-like domain, which has been shown to bind Ca^{2+} and cellular membranes (Hammarberg *et al.*, 2000; Kulkarni *et al.*, 2002), and the C-terminal catalytic domain that contains the catalytic iron in the active site.

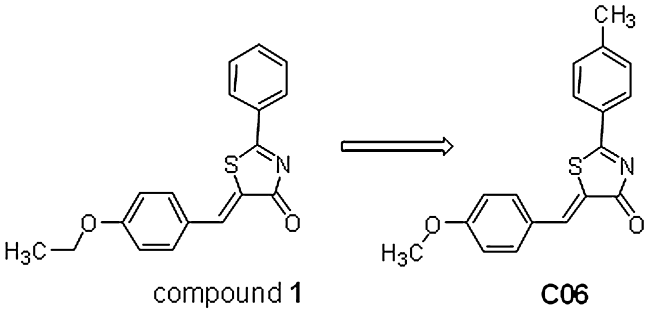

Figure 1

Thiazolinone-based virtual screening hit 1 and the derived compound C06.

Recently, we presented the use of ligand-based virtual screening to identify new inhibitors of 5-LOX product formation (Hofmann *et al.*, 2008). This led to the discovery of a series of thiazolinones represented by 1 [5-(4-ethoxybenzylidene)-2-phenyl-5*H*-thiazol-4-one] (Figure 1) with micromolar 5-LOX inhibitory activity (Hofmann *et al.*, 2008). Here, we present the molecular pharmacological profile of one of the most promising compounds derived from this series (*Z*)-5-(4-methoxybenzylidene)-2-(*p*-tolyl)-5*H*-thiazol-4-one (C06, Figure 1), which is characterized by a novel and unique mode of action different to other established 5-LOX inhibitors.

Methods

Cell preparation

Human polymorphonuclear leukocytes (PMNL) and platelets were freshly isolated from leukocyte concentrates kindly provided by Städtische Kliniken Frankfurt/Höchst. In brief, venous blood was taken from healthy adult donors. Leukocyte concentrates were prepared by centrifugation (4000× *g*, 20 min, 20°C). PMNL and platelets were isolated by dextran sedimentation and centrifugation on Nycoprep cushions (PAA Laboratories, Linz, Austria) as described previously (Werz *et al.*, 2002c). For isolation of PMNL, a hypotonic lysis of erythrocytes was performed. Cells were finally re-suspended in PBS containing 1 $mg \cdot mL^{-1}$ glucose and 1 mM $CaCl_2$ (PGC buffer) (purity >96%). For isolation of platelets, cells were re-suspended in PBS pH 5.4 and centrifuged (1849× *g*, 15 min, 20°C). The cells were re-suspended in PBS/NaCl (PBS pH 5.4, and 0.9% NaCl, 1:1 dilution), centrifuged again (1849× *g*, 10 min, 20°C) and finally re-suspended in PBS pH 5.4.

Cell culture

U937 cells Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) were maintained in RPMI-1640 medium containing 10% fetal calf serum (FCS), 100 $\mu g \cdot mL^{-1}$ streptomycin and 100 $U \cdot mL^{-1}$ penicillin. Rat basophilic leukaemia (RBL)-1 cells (DSMZ) were maintained in RPMI-1640 medium supplemented with

10 mM HEPES, pH 7.4, 10% FCS, 100 μg·mL^{-1} streptomycin, 100 U·mL^{-1} penicillin, 1 mM sodium pyruvate, and nonessential amino acids. RAW264.7 cells American Type Culture Collection (ATCC, Manassas, VA, USA) were grown in DMEM containing 10% FCS, 100 μg·mL^{-1} streptomycin, 100 U·mL^{-1} penicillin and 1 mM sodium pyruvate. Cells were cultured at 37°C and 5% CO_2.

Expression and purification of 5-LOX protein

Escherichia coli BL21 cells were transformed with the plasmid pT3–5LO (Zhang *et al.*, 1992). Recombinant 5-LOX protein was expressed at 22°C and purified from 1-L cultures according to the ATP affinity chromatography procedure as described (Hammarberg *et al.*, 1995; 2000). In brief, *E. coli* was harvested and lysed in 50 mM triethanolamine/HCl, pH 8.0, 5 mM EDTA, soybean trypsin inhibitor (60 μg·mL^{-1}), 1 mM PMSF, 1 mM dithiothreitol and lysozyme (1 mg·mL^{-1}), cooled on ice for 15 min and sonicated (6 × 10 s). Homogenates were centrifuged (10 000× *g*, 15 min), followed by centrifugation at 100 000× *g* (70 min, 4°C). The supernatant was then applied to an ATP-agarose column (Sigma A2767; Deisenhofen, Germany), and the column was eluted as described (Hammarberg *et al.*, 1995; 2000).

Determination of 5-LOX and 15-lipoxygenase-1 (15-LOX-1) product formation in intact cells

For assays of intact cells, 5 × 10^6 or 7.5 × 10^6 freshly isolated PMNL, 3 × 10^6 RBL-1 cells or 2 × 10^7 RAW264.7 cells were re-suspended in 1 mL PGC buffer. After pre-incubation with the test compounds or vehicle (DMSO) at the indicated concentrations for 15 min at 37°C, 5-LOX product formation was stimulated either by pre-incubation with 300 mM NaCl for 3 min and addition of 20 μM AA or stimulated with 2.5 μM calcium ionophore A23187 together with AA at the indicated concentrations. RAW264.7 were stimulated with 10 μM A23187 and 20 μM AA. After 10 min at 37°C, the reaction was stopped with 1 mL methanol. 5-LOX and 15-LOX-1 metabolites were extracted and analysed by HPLC as described (Rödl *et al.*, 2011). The formation of 5-LOX metabolites, including LTB_4 and its all-trans isomers, 5(*S*),12(*S*)-dihydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid [5(*S*),12(*S*)-diHETE], and 5(*S*)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid [5-H(p)ETE], but not the cysteinyl LTs (LTC_4, D_4 and E_4) and oxidation products of LTB_4, was expressed as ng 5-LOX products per 10^6 cells. Activity of 15-LOX-1 was measured by formation of 15(*S*)-hydro(pero)xy-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid. This activity was derived from contaminating eosinophils expressing 15-LOX-1 (this source of 15-HETE was confirmed using a selective 15-LOX-1 inhibitor) (Rai *et al.*, 2010) (data not shown). Data (mean ± SE; $n \geq 3$) are expressed as percentage of control (DMSO).

Determination of 12-lipoxygenase (12-LOX) product formation in intact platelets

For determination of 12-LOX product formation, 10^8 freshly isolated human platelets were re-suspended in 1 mL PGC buffer. After pre-incubation with test compound or vehicle (DMSO) at the indicated concentrations for 15 min at 20°C, 12-LOX product formation was stimulated by addition of 10 μM AA. After 10 min at 37°C, the reaction was stopped with 1 mL methanol. 12-LOX metabolites [12(*S*)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (12-H(p)ETE)] were extracted and analysed by HPLC as described for 5-LOX. Data (mean ± SE; $n \geq 3$) are expressed as percentage of control (DMSO).

Determination of 15-lipoxygenase-2 (15-LOX-2) product formation

0.5 U (i.e. 2 μg) human recombinant 15-LOX-2 was added to 1 mL PBS pH 7.4. After pre-incubation with test compound or vehicle (DMSO) at the indicated concentrations for 15 min on ice, 15-LOX-2 product formation was stimulated with 100 μM AA. After 10 min at 37°C, the reaction was stopped with 1 mL methanol. The 15-LOX-2 metabolites [15(*S*)-hydro(pero)xy-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid] were extracted and analysed by HPLC as described for 5-LOX. Data (mean ± SE; $n \geq 3$) are expressed as percentage of control (DMSO).

Determination of 5-LOX product formation in cell-free systems

5-LOX product formation in cell-free assays was performed as described by Hofmann *et al.*, (2011). In brief, freshly isolated PMNL or harvested rodent cells were sonicated and the whole homogenate was centrifuged (100 000× *g*, 70 min, 4°C) to yield the 100 000× *g* supernatant fraction (S100). For determination of 5-LOX activity, cell homogenate or S100 corresponding to 7.5 × 10^6 PMNL, 5 × 10^6 RBL-1 or 2 × 10^7 RAW264.7 cells was used and additional reagents were added as indicated [e.g. phosphatidylcholine (PC)]. For determination of activity of partially purified 5-LOX from *E. coli*, ~1 μg 5-LOX protein (generating ~1000 ng 5-LOX products) was used. Formed metabolites were analysed by HPLC as described for intact cells. Data (mean ± SE; $n \geq 3$) are expressed as percentage of control (DMSO).

Determination of recombinant COX-2 activity

Inhibitory activity of C06 on recombinant human COX-2 was assayed with a COX Inhibitor Screening Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's protocol. All samples were tested in triplicate. Data (mean ± SE; $n = 2$–3) are expressed as percentage of control (DMSO).

PPAR assay

C06 was evaluated for activation of PPAR subtypes by a reporter gene assay using Cos7 cells described earlier (Derksen *et al.*, 2006). A detailed description can be found in the supporting information.

In vitro *cytosolic phospholipase A_2 ($cPLA_2$) activity assay*

Inhibition of AA release from 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine (PAPC) vesicles by $cPLA_2$ was evaluated as described earlier (Hoffmann *et al.*, 2010). A detailed description can be found in the supporting information.

In vitro *cell viability/cytotoxicity assay*

Cytotoxicity of C06 was determined in PMNL and in a standard cytotoxicity assay. For measurement of cytotoxicity of

C06 in PMNL, 5 × 10^6 PMNL in PGC buffer were incubated with test compounds or vehicle (DMSO) at the indicated concentrations for 30 min at 37°C. Afterwards, cell viability was measured by Trypan blue exclusion. In addition, the WST-1 Assay (Roche Diagnostic, Mannheim, Germany) was used to determine cell viability after treatment with test compounds. U937 cells, which were seeded at a density of 10^4 cells per well, were treated with increasing concentrations of test compound for 48 h. Cell viability was assessed according to the distributor's protocol using a microplate reader (infinite M200, Tecan Group Ltd, Crailsheim, Germany). MK886 was taken as reference compound with known cytotoxicity (Fischer *et al*., 2010). All samples were tested in triplicate. Data (mean ± SE; $n = 3$) are expressed as percentage of control (DMSO).

Statistical analysis

Data are expressed as mean ± SE All IC_{50} values obtained from measurements at three to five different concentrations of the compounds in three to five independent experiments and are approximations determined by graphical analysis (linear interpolation between the points at 50% activity) using SigmaPlot2004 (Systat Software Inc., Chicago, IL, USA). Data were subjected to one-way ANOVA coupled with Tukey's post-tests for multiple comparisons.

Materials

Compound C06 (*Z*)-5-(4-methoxybenzylidene)-2-(*p*-tolyl)-5*H*-thiazol-4-one was synthesized according to Zayed *et al*. (1985) as described previously (Hofmann *et al*., 2011). The structure was confirmed by ^1H and ^{13}C-NMR by mass spectrometry (ESI), and by X-ray investigation; the purity (>98%) was checked by elemental analysis.

Hyperforin was a generous gift by Schwabe AG (Karlsruhe, Germany). MK886, AA, ionophore A23187, DMSO, lipopolysaccharide, Trypan blue and PC were from Sigma, HPLC solvents from Merck (Darmstadt, Germany). The maltose-binding protein (MBP)-5LO1-128 was expressed and purified as described (Michel *et al*., 2008). Human recombinant 15-LOX-2 was obtained from Cayman Chemical, RPMI and DMEM medium, penicillin, streptomycin, glutamine and sodium pyruvate from PAA Laboratories GmbH (Pasching, Germany), nonessential amino acids from Sigma, HEPES buffer from Gibco (Grand Island, NY, USA) and FCS was obtained from Biochrom AG (Berlin, Germany). Human transforming growth factor-β (TGF-β) was purified from outdated platelets as described (Werz *et al*., 1996).

Results

Thiazolinone derivative C06 inhibits 5-LOX in different assay systems by a non-cytotoxic mechanism

As it is frequently observed that the potency of 5-LOX inhibitors depends on the assay conditions and/or experimental settings, the effectiveness of C06 was elucidated both in a cell-based system using human PMNL and cell-free assays utilizing PMNL homogenates, S100 preparations of the homogenates and partially purified recombinant 5-LOX. IC_{50} values ranged from ~0.66 μM in intact PMNL and PMNL homogenates to ~0.3 μM in cell-free S100 and partially purified recombinant 5-LOX (Figure 2). In our standard assay, exogenous AA (20 μM) was added to overcome any limitation of supply of endogenous substrate, mediated by $cPLA_2$.

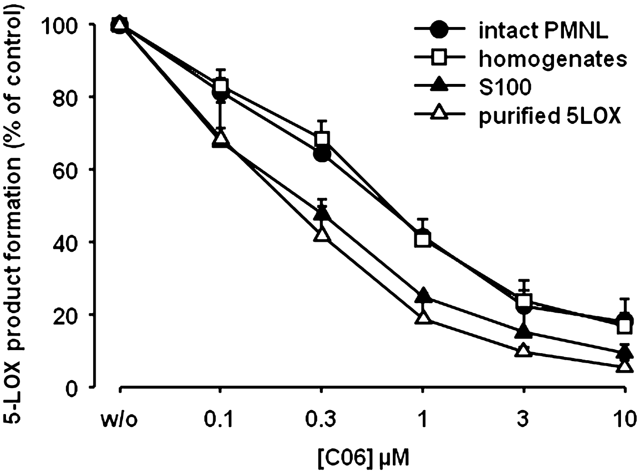

Figure 2

Inhibition of 5-LOX activity by C06 in different assay systems. For determination of 5-LOX product formation in intact cells, freshly isolated PMNL (5 × 10^6) were re-suspended in 1 mL PGC and C06 or vehicle (DMSO) was added at the indicated concentrations. After 15 min at 37°C, cells were stimulated with 2.5 μM calcium ionophore A23187 and AA (20 μM), incubated for another 10 min, and 5-LOX product formation was determined. For determination of 5-LOX product formation in cell-free assays, homogenates or S100 corresponding to 7.5 × 10^6 PMNL or 1 μg partially purified human recombinant 5-LOX were pre-incubated in 1 mL PBS pH 7.4 plus 1 mM EDTA and 1 mM ATP with C06 at the indicated concentrations or vehicle (DMSO) at 4°C. After 10 min, samples were pre-warmed at 37°C for 30 s, and 5-LOX product formation was started by addition of 2 mM $CaCl_2$ and 20 μM AA. After incubation for 10 min, 5-LOX product formation was determined. Data (mean ± SE, $n \geq 3$) are expressed as percentage of control (DMSO). 5-LOX product formation in absence of C06 (100%, control) was 203 ± 12 (intact PMNL), 165 ± 19 (PMNL homogenates) and 138 ± 27 (PMNL S100) ng per 10^6 cells and 407 ± 43 ng per μg partially purified 5-LOX.

To exclude the possibility that the decrease in 5-LOX product formation in intact PMNL was due to cytotoxicity of C06, we measured cell viability in PMNL after incubation with C06 for 30 min. Additionally, we performed a WST-1 cytotoxicity assay with U937 cells. In both assays, cell viability was not impaired after treatment with C06 at relevant concentrations (Figure 3).

Inhibition of 5-LOX by C06 is independent of the redox tone and the MAPK pathway

The efficacy of many 5-LOX inhibitors strongly depends on the mode of cellular 5-LOX activation making prediction of the effects *in vivo*, less reliable. Previous studies showed that the potency of non-redox-type 5-LOX inhibitors was higher in ionophore-stimulated PMNL where 5-LOX activity is evoked by elevation of intracellular Ca^{2+} , compared with 5-LOX stimulation initiated by cellular stress, such as hyper-

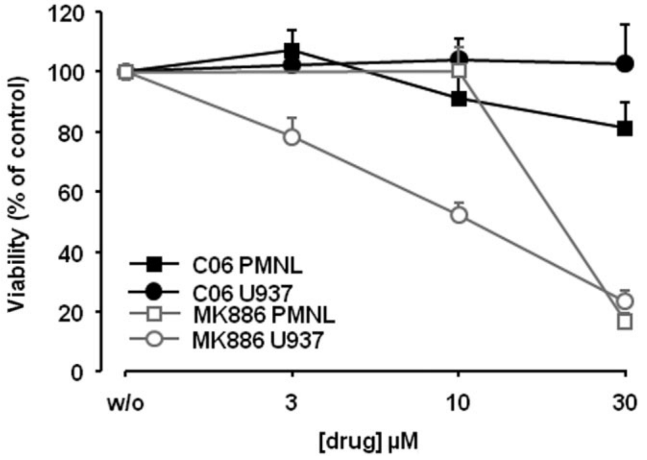

Figure 3

Cell viability of PMNL and U937 cells after incubation with C06. For measurement of cytotoxicity of C06 in PMNL, 5 × 10^6 PMNL in PGC buffer were incubated with test compounds or vehicle (DMSO) at the indicated concentrations for 30 min at 37°C. Cell viability was measured by Trypan blue exclusion. U937 cells were seeded at a density of 10^4 cells per well and treated with increasing concentrations of inhibitor for 48 h. Cytotoxicity was assayed using a WST-1 assay. MK886 was used as cytotoxic reference compound in both assays. Data (mean ± SE, $n = 3$) are expressed as percentage of untreated control cells.

osmotic shock by addition of 300 mM NaCl, and involving p38 MAPK-dependent phosphorylation events (Fischer *et al.*, 2003). Moreover, non-redox-type 5-LOX inhibitors require glutathione peroxidase activity for inhibition of 5-LOX as elevated peroxide levels impair their efficacy (Werz *et al.*, 1998). *In vivo*, there is an enhanced formation of activated oxygen species at inflammatory sites that are involved in 5-LOX activation in stimulated leukocytes. Therefore, it is important to challenge new inhibitors under these conditions. For C06, we could observe almost identical IC_{50} values in intact PMNL, where lipid hydroperoxide levels are low, and in PMNL homogenates, where peroxide levels are high (Werz *et al.*, 1998) (Figure 2). In the same way, C06 activity was unchanged if PMNL homogenates were supplemented with 1 mM glutathione (GSH) to reconstitute glutathione peroxidase activity by elevating the co-substrate concentration (Figure 4A). In contrast to non-redox-type 5-LOX inhibitors (Werz *et al.*, 1998), 5-LOX inhibition by C06 was not improved under reducing conditions. Additionally, no loss of inhibitory activity was observed in PMNL stimulated by 300 mM NaCl (IC_{50} = 0.2 µM), the potency was even slightly higher compared to stimulation with raised intracellular Ca^{2+} (IC_{50} = 0.7 µM) (Figure 4B).

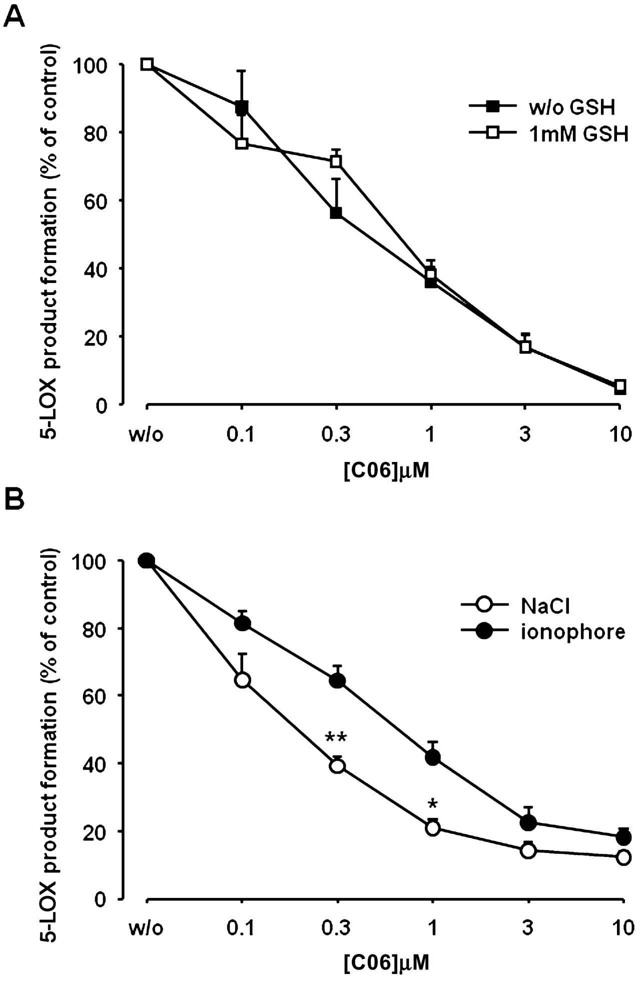

Figure 4

Effects of GSH and cellular stress on 5-LOX inhibition by C06. (A) Homogenates, corresponding to 7.5 × 10^6 neutrophils, were pre-incubated in 1 mL PG-buffer plus 1 mM EDTA and 1 mM ATP with C06 or vehicle (DMSO) at 4°C. GSH (1mM) was added and after 5–10 min, samples were pre-warmed at 37°C for 30 s and 5-LOX product formation was started by addition of 2 mM $CaCl_2$ and 20 µM AA. After 10 min, 5-LOX product formation was determined. (B) Freshly isolated PMNL (5 × 10^6) were re-suspended in 1 mL PGC buffer and C06 or vehicle (DMSO) was added at the indicated concentrations. After 12 min at 37°C, cells were either pre-incubated with 300 mM NaCl for 3 min and AA (20 µM) was added or were stimulated after 15 min at 37°C cells with 2.5 µM calcium ionophore A23187 together with AA (20 µM). After incubation for 10 min, 5-LOX product formation was determined. Data (mean ± SE, $n \geq 3$) are expressed as percentage of control (DMSO). 5-LOX product formation in absence of C06 (100%, control) was 75 ± 7 (w/o GSH), 101 ± 4 (1 mM GSH), 68 ± 16 (NaCl) and 184 ± 37 (ionophore) ng per 10^6 cells. $^*P \leq 0.05$, significantly different from cells stimulated with NaCl in presence of C06.

C06 is a selective 5-LOX inhibitor

We investigated whether C06 shows pleiotropic effects regarding several AA binding proteins, including other forms of LOX (12-LOX, 15-LOX-1 and 15-LOX-2), PPARs, $cPLA_2$ and COX-2. As PMNL preparations (purity >96%) contain eosinophils expressing 15-LOX-1, we analysed the effects of C06 on the concomitant formation of 15-H(p)ETE. The effect on 15-LOX-2 was analysed using the purified human recombinant protein, and for measurement of 12-LOX inhibition, isolated platelets were used. C06 failed to inhibit 15-LOX-1, 15-LOX-2 and 12-LOX product formation up to 10 µM (Figure 5). The increase of 15-H(p)ETE production could be

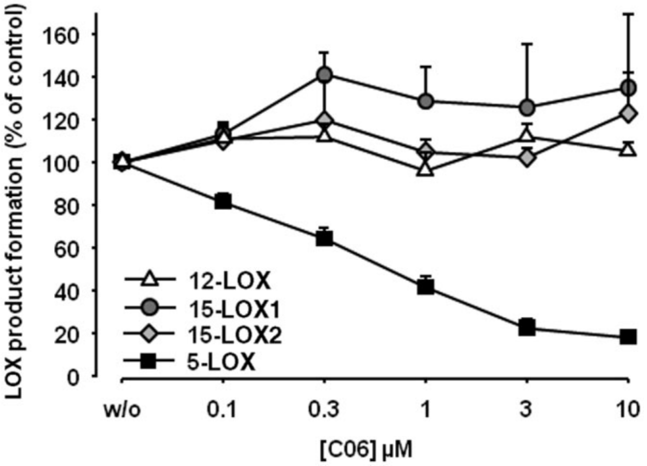

Figure 5

Selectivity profile of C06. For determination of 5-LOX and 15-LOX-1 (12-LOX) product formation, freshly isolated PMNL (5 × 10^6) (10^8 freshly isolated human platelets) were re-suspended in 1 mL PGC buffer. For determination of 15-LOX-2 product formation, 0.5 U human recombinant 15-LOX-2 was added to 1 mL PBS pH 7.4. After pre-incubation with C06 at the indicated concentrations or vehicle (DMSO), samples were stimulated with 2.5 µM calcium ionophore A23187 together with 20 µM AA (12-LOX: 10 µM AA only; 15-LOX-2: 100 µM AA only). After 10 min at 37°C, LO product formation was determined. Data (mean ± SE, $n \geq 3$) are expressed as percentage of control (DMSO). LO product formation in absence of C06 (100%, control) was 197 ± 24 (5-LOX) and 21 ± 5 (15-LOX-1) ng per 10^6 cells, 1446 ± 42 ng per 10^8 cells (12-LOX) and 275 ± 12 ng per 0.5 U (15-LOX-2).

ascribed to a shunt of substrate to 15-LOX-1 when 5-LOX is inhibited at least in our experimental setting where exogenous AA is present.

Moreover, no PPAR activation was observed up to 30 µM C06 in an established reporter-gene assay using Cos7 cells (relative activation of PPAR-α: no activation, PPAR-δ: no activation, PPAR-γ: 27 ± 11%; control: 1 µM pioglitazone = 100%).

To exclude the possibility that C06 inhibited cPLA$_2$, we assessed the effects of the compound on the release of AA from liposomes or Triton-X micelles by purified cPLA$_2$. Liposomes were composed of PAPC, which is a major substrate for cPLA$_2$ in intact platelets (Purdon and Smith, 1985; Purdon *et al.*, 1987). Concentrations of 1 µM and 10 µM of C06 did not inhibit cPLA$_2$ in PAPC-Triton-X-100 micelles [residual activity (RA): 95 ± 6% and 98 ± 13% respectively]. In PAPC liposomes, marginal inhibition at 1 µM and 10 µM C06 could be detected (RA: 89 ± 5% and 73 ± 3% respectively).

No inhibition of recombinant human COX-2 by C06 at a concentration of 5 µM was observed (RA: 114 ± 17%; control 1 µM rofecoxib, RA: 18 ± 8%). The parent compound of C06, compound 1, also failed to inhibit COX-2 (Hofmann *et al.*, 2008).

Activity of 5-LOX in RBL-1 cells and mouse macrophages (RAW264.7) was also inhibited (Table 1), with IC$_{50}$ values within the range of the IC$_{50}$ values recorded for human 5-LOX.

Table 1

IC$_{50}$ values for inhibition of 5-LOX product formation in intact cells and cell-free S100 preparations

	5-LOX product formation IC50 (µM)	
	Intact cells	**S100**
RBL-1	0.2	0.6
RAW264.7	1	0.3
Human PMNL	0.66	0.3

The IC$_{50}$ values shown in the Table represent means of three independent experiments. 5-LOX product formation in control was 138 ± 18 (intact RBL-1), 369 ± 57 (RBL-1 S100), 70 ± 10 (intact RAW264.7) and 112 ± 37 (RAW264.7 S100) ng per 10^6 cells.

Inhibition of 5-LOX by C06 is not affected by high amounts of exogenous AA

For further analysis of the inhibitory molecular mechanism of C06, we tested whether increasing substrate concentrations may affect the potency of C06. Similar 5-LOX inhibition was observed after addition of AA between 2 µM and 20 µM, resulting in IC$_{50}$ values of approximately 0.8 µM (Figure 6A). Using only endogenous substrate, there was a slight shift to IC$_{50}$ = 0.2 µM. This could be due to interference with other AA-releasing pathways (e.g. sPLA$_2$), although we could not detect cPLA$_2$ inhibition.

We used the known competitive 5-LOX inhibitor AA-861 (Yoshimoto *et al.*, 1982) as a control for a competitive inhibitor (Figure 6B). As expected, increasing AA concentrations led to a 30-fold increase of the IC$_{50}$ values from 0.04 µM, 0.2 µM and 0.8 µM to 1.2 µM for the respective AA concentrations (Figure 6B).

Plotting 5-LOX product formation versus substrate concentration at constant inhibitor concentration, it can be seen that the proportion of 5-LOX products formed after incubation with 0.3 µM AA-861 ranged from around 11% (0 µM AA) to 86% (20 µM AA), whereas for C06, this pronounced increase is not observed (18% vs. 30%), suggesting binding to a site different from any fatty acid binding sites of 5-LOX.

PC marginally impairs the potency of C06

The C2-like domain of 5-LOX binds Ca^{2+} and PC (Hammarberg *et al.*, 2000; Kulkarni *et al.*, 2002) and thus targets 5-LOX to the nuclear membrane (Chen and Funk, 2001; Kulkarni *et al.*, 2002). PC binding to 5-LOX and its stimulatory effects require three tryptophan residues (W13, W75 and W102) at the C2-like domain (Kulkarni *et al.*, 2002). For the 5-LOX inhibitor, hyperforin (Albert *et al.*, 2002; Feisst and Werz, 2004), there is strong evidence for its interaction with the C2-like domain of 5-LOX (Feisst *et al.*, 2009). In this regard, a markedly impaired potency of hyperforin in PMNL homogenates, where PC-containing membranes are present, or after exogenous addition of PC was observed (Feisst *et al.*, 2009). To assess a possible interference of C06 with the C2-like domain of 5-LOX, we tested whether PC could impair the potency of this compound. Notably, the inhibitory activity of

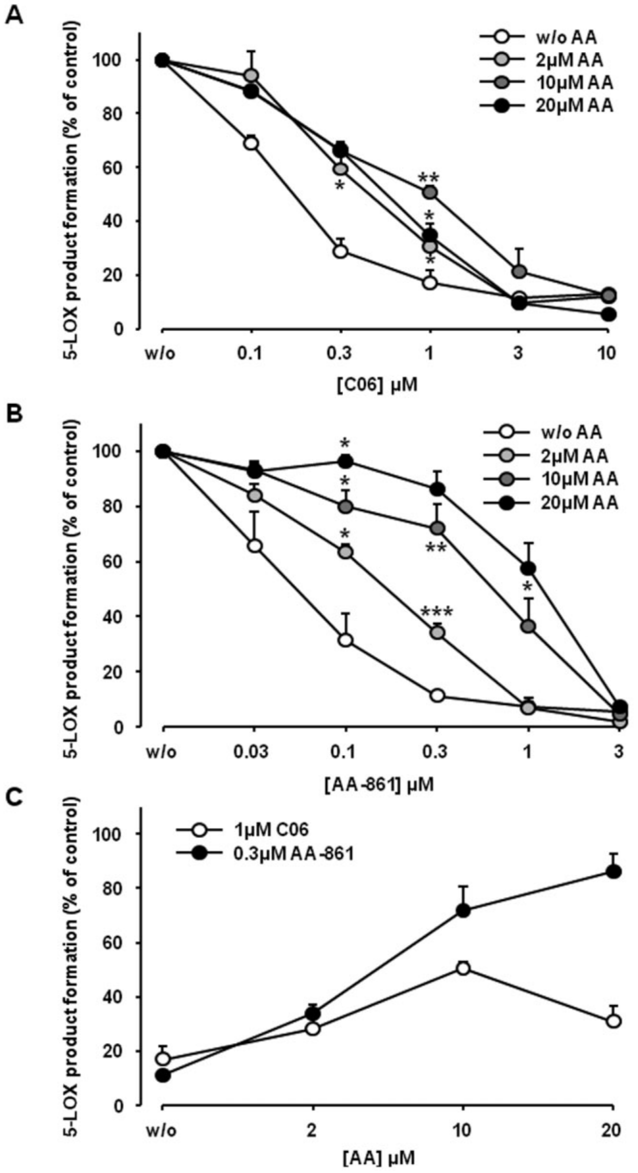

Figure 6

Inhibition of 5-LOX by C06 using different substrate concentrations. Freshly isolated PMNL (7.5×10^6) were re-suspended in 1 mL PGC buffer and (A) C06, (B) AA-861 or vehicle (DMSO) was added at the indicated concentrations. After 15 min at 37°C, cells were stimulated with 2.5 μM calcium ionophore A23187 together with arachidonic acid at the indicated concentrations. After 10 min incubation, 5-LOX product formation was determined. Data (mean ± SE, $n \geq 3$) are expressed as percentage of control (DMSO). 5-LOX product formation in absence of C06 (100%, control) was 21 ± 2 (0 μM AA), 37 ± 5 (2 μM AA), 103 ± 12 (10 μM AA) and 164 ± 12 (20 μM AA) ng per 10^6 cells. $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$, significantly different from the next lower AA concentration. (C) Plot of 5-LOX products formed at constant inhibitor concentration (1 μM C06, 0.3 μM AA-861) with increasing substrate concentration.

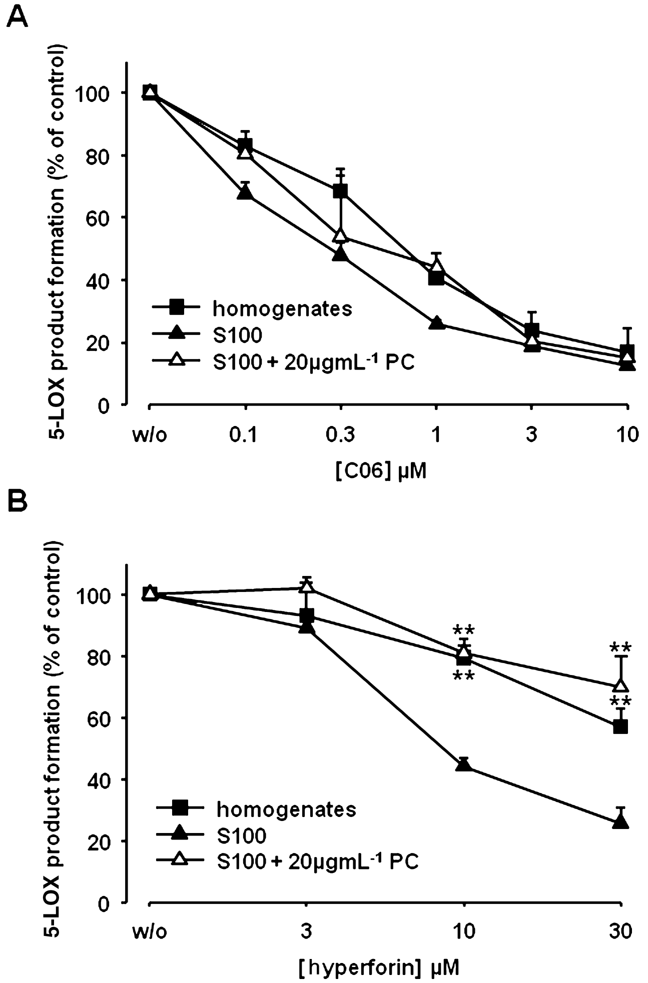

Figure 7

Influence of phosphatidylcholine (PC) on 5-LOX inhibition by C06. Homogenates and S100 from 7.5×10^6 PMNL were pre-incubated in 1 mL PBS pH 7.4 plus 1 mM EDTA and 1 mM ATP with (A) C06 or (B) hyperforin at the indicated concentrations or vehicle (DMSO) at 4°C with or without PC (20 μg·mL^{-1}) as indicated. After 10 min, samples were pre-warmed (37°C) for 30 s and 5-LOX product formation was started by addition of 2 mM $CaCl_2$ and 20 μM AA. After 10 min, 5-LOX product formation was determined. Data (mean ± SE, $n \geq 3$) are expressed as percentage of control (DMSO). 5-LOX product formation in absence of C06 (100%, control) was 108 ± 7 (homogenates), 111 ± 18 (S100) and 129 ± 19 (S100, PC) ng per 10^6 cells. $^{**}P \leq 0.01$, significantly different from S100 samples in presence of C06.

C06 in phospholipid-free PMNL S100 preparations (Hörnig *et al*., 2005) was higher (IC_{50} = 0.3 μM) than in PMNL homogenates or in PMNL S100 supplemented with PC (20 μg·mL^{-1}) (IC_{50} values: both ~0.8 μM) (Figure 7A). The control agent hyperforin exhibited an IC_{50} value of ~7 μM in phospholipid-free S100 preparations, whereas addition of PC (20 μg·mL^{-1}) resulted in an IC_{50} value of >30 μM, similar to its potency in PMNL homogenates (Figure 7B). Taken together, PC binding hardly affected the inhibitory activity of C06 so that its mode of action seems to be clearly different from that of hyperforin.

Excess of 5-LOX C2-like domain does not impair the potency of C06

To further investigate a possible binding of C06 to the C2-like domain of 5-LOX, we analysed the effect of increasing amounts of C2-like domain on the 5-LOX inhibitory potency of C06 in an established C2-like domain competition assay. We could not detect direct interference of C06 with the C2-like domain of 5-LOX (supporting information, Figure S1).

Discussion and conclusions

In this study, we have assessed a novel 5-LOX inhibitor based on a thiazolinone scaffold, identified by ligand-based virtual screening (Hofmann *et al.*, 2008). Thiazolinones have already been disclosed as mixed COX/LOX inhibitors. In 1994, Unangst *et al.* reported dual 5-LOX/COX inhibition by (*Z*)-5-[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]-methylene]-2-imino-4-thiazolidinone (Unangst *et al.*, 1994). Recently, Geronikaki *et al.* (2008) identified a series of 2-thiazolylimino-5-arylidene-4-thiazolidinones with moderate activity against soybean lipoxygenase and COX-1. Our results confirm the thiazolinone structure as a suitable pharmacophore for 5-LOX inhibition. In addition, C06 exhibits with its 2-phenyl moiety and the distinct substitution pattern a unique and novel scaffold that discriminates it from the known thiazolinones. Notably, for compound 1 (Hofmann *et al.*, 2008) and C06, no inhibition of COX-2 was observed. Thus, it is possible that the 2-phenyl substituent is responsible for selective 5-LOX inhibition. To our knowledge, this structural element has not been described in a 5-LOX/COX inhibitor so far. It also seems to be part of the pharmacophore: we have performed a structure–activity relationship study with 34 derivatives of compound 1 (Hofmann *et al.*, 2011) revealing the importance of this moiety for 5-LOX inhibitory activity.

The 5-benzylidene-2-phenyl-thiazolinone, C06, represents a promising novel 5-LOX inhibitor with IC_{50} values in the nanomolar concentration range in intact cells and cell-free assays. In contrast to many non-redox-type inhibitors such as ZM230487 and L-739.010 (Fischer *et al.*, 2003), C06 showed full inhibitory potency when 5-LOX is activated by phosphorylation. Also, the redox tone in the cell did not affect its potency. This represents a clear advantage over known non-redox-type 5-LOX inhibitors for which a loss of activity is often observed if the peroxide concentration is high or the enzyme is activated by phosphorylation. This is an important finding as chronic inflammatory processes are linked to oxidative stress and increased intracellular peroxide levels, which, in turn, could explain the reported lack of efficacy of non-redox-type 5-LOX inhibitors in patients (Nasser *et al.*, 1994; Turner *et al.*, 1996). Furthermore, as C06 showed similar inhibitory potency in intact cells to that found in cell-free systems, the involvement of FLAP in the inhibitory mechanism of C06 can be excluded. Thus, C06 might be more effective than the FLAP inhibitors which are known to be less potent at high AA levels in the cell. Finally, based on the structure of C06, iron chelating and reducing properties are unlikely for the 5-LOX inhibitory mechanism of the drug. Again, this can be beneficial as most redox-type inhibitors lack suitable oral bioavailability, exhibit only poor selectivity for 5-LOX and thus can cause severe side effects, such as methemoglobin formation, due to interference with other biological redox systems or by the production of reactive radical species (McMillan and Walker, 1992; Ford-Hutchinson *et al.*, 1994). Also C06 inhibited 5-LOX via non-cytotoxic mechanisms. This is an advantage over several other 5-LOX inhibitors, known to exhibit cytotoxicity due to off-target effects (Fischer *et al.*, 2010).

The high selectivity of C06 for human and rodent 5-LOX compared with other AA binding proteins and several LOX isoforms suggests that possible off-target effects are unlikely. As a result, the compound also may represent a useful pharmacological tool in experimental models of inflammation to study the role of 5-LOX.

Confirmation of our findings *in vivo* would have added considerable strength to our hypothesis. However, although we went to considerable effort to address this point, the low solubility of the lipophilic compound meant that we were not able to obtain significant plasma and tissue levels of C06 for effective target inhibition. However, we hope to produce a more water-soluble compound by synthesizing and evaluating several derivatives based on the scaffold with improved solubility characteristics.

In terms of a possible binding site for C06, we would propose allosteric binding distinct from fatty acid binding sites and the C2-like domain of 5-LOX. As excess of AA did not impair the potency of C06, the drug is unlikely to bind to the active site or to a hypothetical second fatty acid binding site which maybe located in the C2-like domain, as proposed by Romanov *et al.* (2006). They performed photoaffinity labelling studies of rabbit 12/15-LOX with lipoxygenase substrates and identified a possible second fatty acid binding site within the C2-like domain. Consistent with this, the addition of membrane-mimicking PC did not impair C06 efficacy, in contrast to hyperforin whose 5-LOX inhibitory potency was decreased in presence of PC. It should be mentioned that another phospholipid, phosphatidylethanolamine, did not interfere with 5-LOX inhibition by hyperforin (Feisst *et al.*, 2009), making non-specific effects by phospholipid-vesicles, such as trapping the inhibitor, less likely. Finally, the C2-like domain did not compete with 5-LOX in C06 binding (Figure S1), suggesting that C06 should not interact with this domain. Studies to resolve the binding mode to further investigate our hypothesis and to allow for structure-based lead optimization are ongoing in our lab.

Compared with zileuton, the only 5-LOX inhibitor in clinical use, which inhibits 5-LOX activity in various assay systems with IC_{50} of 0.5–1 μM (Carter *et al.*, 1991), C06 is up to fivefold more potent. C06 might open up a novel approach for 5-LOX inhibition via a possible mode of action at an allosteric site.

In summary, using virtual screening, we have identified a novel direct 5-LOX inhibitor with activity in the nM concentration range, both in whole cells and cell-free systems. C06 acts by a novel and unique mode of action, different to other established 5-LOX inhibitors and thus might lack several disadvantages of the 5-LOX inhibitors developed so far, including FLAP inhibitors, iron ligand and redox- and non-redox-type inhibitors. Furthermore, C06 exhibits high selectivity for 5-LOX and might bind to an allosteric binding site. This distinct pharmacological profile is promising and encourages further investigations and structure optimization of this novel 5-LOX inhibitor.

Acknowledgement

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Conflict of interest

The authors state no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Response of C06 to C2-like domain competition assay. Increasing amounts of MBP-5LO1-128 (adjusted with γ-globulin to a final protein concentration of 300 μg·mL^{-1}) were added to recombinant 5-LOX (0.5 μg·mL^{-1}). The inhibitory potency of 1 μM C06 [expressed as percentage of control (w/o C06)] with and without an excess of C2-like domain was assayed under standard assay conditions (0.5 μg 5-LOX protein in 1 mL PBS pH 7.4 containing 1 mM EDTA, 1 mM ATP, 2 mM $CaCl_2$ and 20 μM AA). Data (mean ± SE, $n = 3$) are expressed as percentage of control (DMSO).