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Molecular characterization of **EP6**—A novel imidazo[1,2-*a*]pyridine based direct 5-lipoxygenase inhibitor

Joanna M. Wisniewska [a], Carmen B. Rödl [a], Astrid S. Kahnt [a], Estel.la Buscató [a], Sandra Ulrich [a], Yusuf Tanrikulu [b], Janosch Achenbach [a], Florian Rörsch [c], Sabine Grösch [c], Gisbert Schneider [d], Jindrich Cinatl Jr.[e], Ewgenij Proschak [a], Dieter Steinhilber [a], Bettina Hofmann [a,*]

[a] *Institute of Pharmaceutical Chemistry, ZAFES/LiFF, Goethe-University Frankfurt, Max-von-Laue-Str. 9, D-60438 Frankfurt/Main, Germany*
[b] *Institute of Organic Chemistry and Chemical Biology, ZAFES/CMP, Goethe-University Frankfurt, Siesmayerstr. 70, D-60323 Frankfurt/Main, Germany*
[c] *Institute of Clinical Pharmacology, ZAFES/LiFF, Goethe-University Frankfurt, Theodor-Stern-Kai 7, D-60590 Frankfurt/Main, Germany*
[d] *Institute of Pharmaceutical Sciences, ETH Zurich, Wolfgang-Pauli-Str. 10, CH-8093 Zurich, Switzerland*
[e] *Institute of Medical Virology, ZAFES/LiFF, Goethe-University Frankfurt, Paul-Ehrlich-Str. 40, D-60596 Frankfurt/Main, Germany*

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ABSTRACT

5-Lipoxygenase (5-LO) is a crucial enzyme of the arachidonic acid (AA) cascade and catalyzes the formation of bioactive leukotrienes (LTs) which are involved in inflammatory diseases and allergic reactions. The pathophysiological effects of LTs are considered to be prevented by 5-LO inhibitors. In this study we present cyclohexyl-[6-methyl-2-(4-morpholin-4-yl-phenyl)-imidazo[1,2-*a*]pyridin-3-yl]-amine (**EP6**), a novel imidazo[1,2-*a*]pyridine based compound and its characterization in several *in vitro* assays. **EP6** suppresses 5-LO activity in intact polymorphonuclear leukocytes with an IC_{50} value of 0.16 μM and exhibits full inhibitory potency in cell free assays (IC_{50} value of 0.05 μM for purified 5-LO). The efficacy of **EP6** was not affected by the redox tone or the concentration of exogenous AA, characteristic drawbacks known for the class of nonredox-type 5-LO inhibitors. Furthermore, **EP6** suppressed 5-LO activity independently of the cell stimulus or the activation pathway of 5-LO contrary to what is known for some nonredox-type inhibitors. Using molecular modeling and site-directed mutagenesis studies, we were able to derive a feasible binding region within the C2-like domain of 5-LO that can serve as a new starting point for optimization and development of new 5-LO inhibitors targeting this site. **EP6** has promising effects on cell viability of tumor cells without mutagenic activity. Hence the drug may possess potential for intervention with inflammatory and allergic diseases and certain types of cancer including leukemia.

1. Introduction

Leukotrienes (LTs), inflammatory mediators formed from arachidonic acid (AA), are associated with inflammatory and allergic diseases like asthma and allergic rhinitis, as well as cardiovascular diseases (atherosclerosis) and certain types of cancer [1–4]. 5-Lipoxygenase (5-LO) catalyzes oxygenation of AA to 5(*S*)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HpETE) and further dehydration to the unstable epoxide leukotriene A_4 (LTA_4). The subsequent conversion of LTA_4 by LTA_4 hydrolase leads to leukotriene B_4 (LTB_4), and the conjugation of LTA_4 with GSH by LTC_4 synthase yields the cysteinyl leukotriene C_4 (LTC_4) [2,5]. Due to the role of 5-LO as a key enzyme in the biosynthesis of all LTs, 5-LO inhibitors are of therapeutic value for the treatment of asthma, allergic rhinitis, atherosclerosis as well as certain types of cancer [3,6]. Also recently, it was found that the ALOX5 gene is a critical regulator for leukemia stem cells in BCR-ABL-induced chronic

Abbreviations: 5-H(p)ETE, 5(*S*)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid; 12-H(p)ETE, 12(*S*)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid; 15-H(p)ETE, 15(*S*)-hydro(pero)xy-5,8,11-*cis*-13-*trans*-eicosatetraenoic; 5(*S*),12(*S*)-diHETE, 5(*S*),12(*S*)-dihydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid; A, alanine; AA, arachidonic acid; Ada, adenosine deaminase; CML, chronic myeloid leukemia; COX, cyclooxygenase; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; FLAP, 5-LO activating protein; fMLP, N-formyl-methionine-leucine-phenylalanine; LC–MS/MS, liquid chromatography coupled with tandem mass spectrometry; LDH, lactate dehydrogenase; LO, lipoxygenase; LT, leukotriene; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MBP, maltose binding protein; mPGES-1, microsomal prostaglandin E_2 synthase-1; PARP, poly ADP-ribose polymerase; PB, phosphate buffer pH 7.4; PC, phosphatidylcholine; PDB, Protein Data Bank; PG buffer, PBS containing 1 mg/ml glucose; PGC buffer, PBS containing 1 mg/ml glucose and 1 mM $CaCl_2$; PLIF, Protein Ligand Interaction Fingerprints; PMNL, polymorphonuclear leukocytes; PMSF, phenylmethylsulfonyl fluoride; RPE, retinal pigment epithelium; RT, room temperature; S100, 100 000 × *g* supernatant; SA, sodium arsenite; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; W, tryptophan; WT, wild-type; Y, tyrosine.

* Corresponding author. Tel.: +49 69 798 29335; fax: +49 69 798 29323.
E-mail address: hofmann@pharmchem.uni-frankfurt.de (B. Hofmann).

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myeloid leukemia (CML) [7]. Chen et al. could show, that the use of the 5-LO inhibitor zileuton [8], alone or in combination with imatinib, impairs CML (clinical trial phase I) [7]. Up to now, zileuton is the only 5-LO inhibitor that reached the market in the US for the treatment of asthma.

The reported crystal structure of a stable form of the 5-LO (Protein Data Bank (PDB), http://pdb.org [9], ID: 3o8y [10]), implies a folding of the single polypeptide chain in a two domain structure: a N-terminal regulatory C2-like domain and a C-terminal catalytic domain. The C2-like domain, organized in a β-sandwich, is important for membrane binding [11,12] or binding of diacylglycerides [13] and calcium [14], respectively. The binding site for cellular membranes or phosphatidylcholine (PC), serving as stimulating scaffolds for the 5-LO, is defined by three tryptophan residues (W13, W75 and W102) of the C2-like domain [12]. An ATP binding site formed by the C2-like domain was postulated as well [15]. The catalytic domain, primarily consisting of α-helices, contains a non-heme iron responsible for substrate conversion. In the inactive state of the enzyme, the iron is in the ferrous (Fe^{2+}) form; for entering the catalytic pathway an oxidation by hydroperoxides to the ferric (Fe^{3+}) form is necessary [16]. Reducing agents and iron-ligand inhibitors such as zileuton or BWA4C [17] stabilize the ferrous form or chelate with the central iron-atom, thereby suppressing 5-LO activity. These direct 5-LO inhibitors exhibit high inhibitory potency *in vitro*, but show unselective interactions and may interfere with cellular redox reactions [18,19]. Another group of potent and selective 5-LO inhibitors are nonredox-type inhibitors such as Rev-5901 [20], ZD2138 [21] and ZM230487 [22]. This class of inhibitors reduces acute inflammatory responses [18,23], but does not show effects on more chronic processes [24,25]. Furthermore a decreased inhibitory potency of ZM230487 in presence of elevated peroxide tone has been observed [26]. The efficacy of ZM230487 depends on the way of enzyme activation [27]. 5-LO can be activated by an increase of intracellular calcium leading to translocation of 5-LO from the cytosol to the nuclear membrane [28] or by phosphorylation via the p38 mitogen-activated protein kinase (MAPK) and the extracellular signal-regulated kinase (ERK) pathway at two serine residues [29,30]. ZM230487 exhibits impaired 5-LO inhibition after enzyme activation via phosphorylation [27]. Some inflammatory reactions, e.g. allergic asthma, various types of cancer and atherosclerosis are associated with an increased phosphorylation status of the cell [31,32], concluding that nonredox-type inhibitors such as ZM230487 are limited in their benefit. In addition, indirect 5-LO inhibition can regulate the 5-LO product formation. The membrane-bound 5-LO activating protein (FLAP), facilitates the transfer of AA within the nuclear membrane to 5-LO [33]. Targeting FLAP, the 5-LO product formation can be inhibited [34]. FLAP antagonists like MK-886 [35], MK-0591 [36] or AM103 [37] inhibit 5-LO product formation in whole cell preparation and could enter clinical phase II trials for the treatment of inflammatory diseases [38]. Therefore, there is a need for the development of new inhibitors with a different pharmacological profile which potently inhibit the enzyme activity regardless of the mode of enzyme activation. Consequently, potential drug candidates should be screened in different cellular assays where both calcium- and phosphorylation-dependent stimuli are applied.

In this study we evaluate the pharmacological profile of the novel 5-LO inhibitor cyclohexyl-[6-methyl-2-(4-morpholin-4-yl-phenyl)-imidazo[1,2-*a*]pyridin-3-yl]-amine (**EP6**) (Fig. 1A), an analogue of compound **1** and **2** (Fig. 1B) derived from a virtual screening for cyclooxygenase (COX)/5-LO dual inhibitors [39]. Compound **1** and **2**, imidazo[1,2-*a*]pyridin derivatives, inhibit 5-LO product formation in intact polymorphonuclear leukocytes (PMNL) with IC_{50} values of 0.9 μM and 0.6 μM, respectively [39]. Structural optimization led to **EP6**, which exhibits improved inhibitory potency of 5-LO. Herein, we describe the molecular pharmacology of this novel compound with a unique mode of action different to other established 5-LO inhibitors using various *in vitro* test systems.

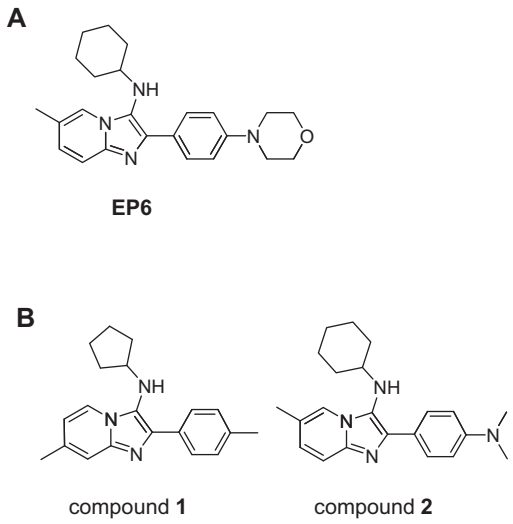

Fig. 1. Chemical structures of (A) **EP6** and (B) compound **1** and **2**.

2. Material and methods

2.1. Chemistry

Compound **EP6** was synthesized according to ref. [40] as described in the supplemental information.

2.2. Materials

AA, calcium ionophore A23187, BWA4C, DMSO, GSH, 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine (PC) and trypan blue solution were purchased from Sigma–Aldrich (Munich, Germany), Rev-5901, zileuton and human recombinant 15-LO2 from Cayman Chemical (Ann Arbor, USA). HPLC solvents were purchased from Merck (Darmstadt, Germany), penicillin, streptomycin and IMDM medium from PAA laboratory GmbH (Pasching, Austria). Fetal calf serum (FCS) was purchased from Biochrom AG (Berlin, Germany), RPMI 1660 medium from Gibco/Invitrogen (Paisley, UK). Fresh blood cell concentrates were provided by Städtische Kliniken Frankfurt-Höchst (Frankfurt, Germany).

2.3. Cell culture

The human leukemic monocyte cells U937 and human cervix carcinoma cells HeLa were purchased from Deutsche Sammlung für Mikroorgansimen und Zellkulturen (DSMZ, Braunschweig, Germany), and mouse leukemic monocyte macrophage cells RAW 264.7 from American Type Culture Collection (ATCC, Manassas, USA). Human retinal pigment epithelium (RPE) cells were isolated as described before [41]. U937, HeLa and RAW 264.7 cells were maintained in RPMI 1640 medium containing 10% FCS, 100 μg/ml streptomycin and 100 U/ml penicillin. RPE cells were maintained in IMDM medium with 20% FCS, L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin. Cells were cultured at 37 °C in an atmosphere containing 5% CO_2.

2.4. Isolation of PMNL and platelets from leukocyte concentrates

PMNL and platelets were freshly isolated from leukocyte concentrates obtained from Städtische Kliniken Frankfurt Höchst

(Frankfurt, Germany). Blood samples were obtained by the hospital with informed consent. In brief, cells were isolated by dextran sedimentation for 30 min, and 10 min centrifugation without deceleration at 800 × *g* on Nycoprep cushions (PAA laboratory GmbH, Pasching, Austria). For PMNL isolation the cells were re-suspended in 10 ml ice-cold water for hypotonic lysis of erythrocytes as described previously [42]. Cells were finally re-suspended in phosphate-buffered saline, pH 7.4 (PBS) containing 1 mg/ml glucose (PG buffer) (purity >96–97%). Platelets were re-suspended in PBS, pH 5.4 and centrifuged at 1849 × *g* for 15 min at room temperature (RT). The cells were re-suspended in PBS/NaCl (PBS, pH 5.4 and 0.9% NaCl, 1:1 dilution) and centrifuged at 1849 × *g* for 10 min at RT. Finally platelets were re-suspended in PBS pH 5.4.

2.5. Site-directed mutagenesis, expression and purification of 5-LO proteins and maltose binding protein (MBP) constructs

The codons for selected residues in plasmid pT3-5-LO [43] were mutated by site-directed mutagenesis polymerase chain reaction yielding the mutated 5-LO plasmids pT3-5-LO-W13/75/102A, pT3-5-LO-Y81A, pT3-5-LO-Y100A, pT3-5-LO-Y81/100A, pT3-5-LO-Y383A or pT3-5-LO-Y81/100/383A as well as in plasmid pBUF-5-LO-C2 [44] for MBP-5-LO fusion proteins yielding the mutated MBP-5-LO-C2 plasmid pBUF-5-LO-Y81/100A. The mutated DNA was confirmed by DNA-sequencing (carried out by Scientific Research and Development GmbH, Bad Homburg, Germany). *Escherichia coli* BL21 were transformed with plasmid pT3-5-LO as wild-type (WT) DNA, pBUF-5-LO-C2 DNA, pBUF1b [44] DNA and mutated DNA. The recombinant 5-LO proteins, 5-LO C2 fusion proteins with MBP or MBP itself were expressed at 22 °C (5-LO-Y81/100/383A at 16 °C) and purified from 1-l cultures according to the ATP affinity chromatography procedure as described previously [14,45] or to the MBP affinity chromatography described by Michel et al. [44]. For experiments investigating the effect of ATP or calcium, the 5-LO protein was further purified via anion exchange chromatography as described previously [46]. In brief, the ATP-eluate (10 ml) was loaded on a ResourceQ 6 ml column (GE Healthcare, Uppsala, Sweden). Buffer A was phosphate buffer (PB) 0.05 mM, pH 7.4 containing 1 mM EDTA, buffer B was buffer A plus 0.5 M NaCl. The elution of the 5-LO was performed in a gradient from 0 to 100% buffer B and the enzyme eluted at about 40% buffer B.

2.6. Determination of 5-LO product formation in intact cells

For assays in intact cells, 5×10^6 or 7.5×10^6 freshly isolated PMNL were re-suspended in 1 ml of PBS, pH 7.4 containing 1 mg/ml glucose and 1 mM $CaCl_2$ (PGC buffer). After pre-incubation with the test compound or vehicle (DMSO) at the indicated concentrations for 12 min at 37 °C, 5-LO product formation was stimulated either by pre-incubation with 300 mM NaCl for 3 min or with 10 μM sodium arsenite (SA) and addition of 20 μM AA, or stimulated with 2.5 μM calcium ionophore A23187 together with exogenous AA at the indicated concentrations after 15 min pre-incubation. For assays with stimulation via N-formyl-methionine-leucine-phenylalanine (fMLP), 7.5×10^6 or 10^7 freshly isolated PMNL were re-suspended in PGC buffer, primed for 15 min with 1 μg/ml lipopolysaccharide (LPS) and 0.2 U/ml adenosine deaminase (Ada) and incubated for 15 min with test compound or vehicle (DMSO). The 5-LO product formation was started by addition of 1 μM fMLP with or without addition of 20 μM AA. For assays with intact mouse leukemic monocyte macrophage cells (RAW 264.7), 1.5×10^7 RAW 264.7 cells were re-suspended in PGC buffer and pre-incubated with the test compound or vehicle (DMSO) at the indicated concentrations for 15 min at 37 °C, 5-LO product formation was stimulated by addition of 5 μM calcium ionophore A23187 together with 20 μM AA. After 10 min at 37 °C, the reaction was stopped with 1 ml of ice-cold methanol. 30 μl of 1 N HCl, 200 ng of prostaglandin B_1 as internal standard and 500 μl of PBS, pH 7.4 were added. 5-LO metabolites were extracted and analyzed by HPLC as described [46]. Metabolites formed via fMLP stimulation without exogenously added AA were measured by LC–MS/MS analysis as described previously [47]. 5-LO product formation was determined as ng of 5-LO products per 10^6 cells, which includes 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). Cysteinyl LTs (LTC_4, D_4 and E_4) and oxidation products of LTB_4 were not determined. Each experiment was performed at least three times. Data (mean ± standard error (S.E.)) are expressed as percentage of control (DMSO).

2.7. Determination of 12-LO and 15-LO1 product formation in intact cells

Determination of 15-LO1 product formation was performed in PMNL preparations as described for 5-LO product formation in intact cells. For determination of 12-LO product formation 1×10^8 freshly isolated platelets were re-suspended in 1 ml of PGC buffer (pH 7.4) and were pre-incubated with the test compound or vehicle (DMSO) at the indicated concentrations for 15 min at 37 °C. 12-LO product formation was stimulated by addition of 10 μM AA. After 10 min at 37 °C, the reaction was stopped with 1 ml of ice-cold methanol. 12-LO products include 12(*S*)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (12-H(p)ETE). 12-HETE and 12-HpETE elute as one major peak. 15-LO1 products, generated from eosinophils expressing 15-LO1, were 15(*S*)-hydro(pero)xy-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid, which elute as one major peak as well. Data (mean ± S.E.; $n \geq 3$) are expressed as percentage of control (DMSO).

2.8. Determination of 5-LO product formation in cell-free systems

PMNL were re-suspended in 1 ml of PBS containing 1 mM EDTA and the protease inhibitors soybean trypsin inhibitor (60 μg/ml), 1 mM phenylmethylsulfonyl fluoride (PMSF), and leupeptin (10 μg/ml), cooled on ice for 10 min, and subsequently, the samples were sonicated for 3 × 10 s (homogenate). To obtain 100 000 × *g* supernatant (S100) the homogenate was centrifuged (100 000 × *g* for 70 min at 4 °C). For determination of 5-LO activity, cell homogenates or S100 corresponding to 7.5×10^6 PMNL were re-suspended in 1 ml of reaction mix (PBS, pH 7.4, 1 mM EDTA, and 1 mM ATP) and additional reagents were added as indicated (e.g. PC, GSH). For cell-free assays of mouse leukemic monocyte macrophage cells (RAW 264.7), 2×10^7 RAW 264.7 cells were prepared as described for PMNL S100 preparations. For determination of the activity of partially purified WT 5-LO, 5-LO-3Y mutant or S100 of WT 5-LO or S100 of 5-LO-3W mutant from *E. coli*, 2–3 μg of 5-LO were dissolved in 1 ml reaction mix. After pre-incubation with the test compounds or vehicle (DMSO) at the indicated concentrations for 15 min at 4 °C, the samples containing either homogenates, S100 or purified 5-LO were pre-warmed for 30 s at 37 °C. The reaction was started after addition of 2 mM $CaCl_2$ and 20 μM AA. After 10 min 5-LO product formation was stopped with 1 ml ice-cold methanol and the formed metabolites were analyzed by HPLC as described for intact cells. Data (mean ± S.E.; $n \geq 3$) are expressed as percentage of control (DMSO).

2.9. Determination of 15-LO2 product formation in cell-free systems

0.5 U human recombinant 15-LO2 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-LO2 product formation was stimulated with 100 μM AA. After 10 min at 37 °C, the reaction was stopped with 1 ml of ice-cold methanol. 15-LO2

metabolites were extracted and analyzed by HPLC as described for intact cells. Data (mean ± S.E.; $n \geq 3$) are expressed as percentage of control (DMSO).

2.10. C2-like domain competition assay

2–3 μg of partially purified 5-LO was dissolved in 1 ml reaction mix (PBS, pH 7.4, 1 mM EDTA, and 1 mM ATP) and increasing amounts of MBP-5-LO-C2, MBP-5-LO-C2 Y81/100A or MBP were added (0–300 μg/ml). γ-Globulin was added to adjust consistent protein concentration of 300 μg/ml. After pre-incubation with 0.3 μM **EP6** or vehicle (DMSO) for 15 min at 4 °C, the samples were pre-warmed for 30 s at 37 °C. The reaction was started after addition of 2 mM $CaCl_2$ and 20 μM AA. After 10 min 5-LO product formation was stopped with 1 ml ice-cold methanol and the formed metabolites were analyzed by HPLC as described for intact cells. For each sample in presence of **EP6**, the sample incubated with vehicle (DMSO) with the same amount of added protein construct was taken as control. Data (mean ± S.E.; $n \geq 3$) are expressed as percentage of control (DMSO).

2.11. COX and mPGES-1 assay

Inhibitory activity of **EP6** on recombinant ovine COX-1 and human COX-2 were assayed with a COX Inhibitor Screening Assay Kit (Cayman Chemical, Ann Arbor, USA) according to the manufacturer's protocol. All samples were tested in triplicate. Data (mean ± S.E.; $n = 3$) are expressed as percentage of control (DMSO).

For investigation of the inhibitory potency of **EP6** on the mPGES-1 enzyme *in vitro*, the microsomal fraction of HeLa cells was prepared. 3×10^6 cells were incubated at 37 °C and after 24 h the medium was removed and the cells where stimulated for 16 h with fresh medium containing 1 ng/ml IL-1β and 5 ng/ml TNFα. After separation into cytosolic and microsomal fraction described previously by Rörsch et al. [48], the microsomal fraction was re-suspended in 100 μl potassium phosphate buffer (0.1 M, pH 7.4), containing 1× complete protease inhibitor cocktail and reduced GSH (2.5 mM), and sonicated (1 × 10 s). The mPGES-1 activity assay was performed as described by Thorén and Jakobsson [49]. Briefly, 0.15 mg/ml protein was incubated with increasing concentrations of the test compounds for 30 min at RT. The reaction was initiated at 4 °C with 20 μM prostaglandin H_2 (Larodan, Malmö, Sweden) and terminated after 1 min by adding a stop solution containing 40 mM iron chloride and 80 mM citric acid. After solid phase extraction the amount of produced prostaglandin E_2 was measured by LC–MS/MS analysis as described previously [50].

2.12. In vitro cell viability assay

The WST-1 assay (Roche Diagnostic GmbH, Mannheim, Germany) was used to determine cell viability after treatment with test compound. U937 cells were seeded in 96-well plates at a density of 10^4 cells/well and treated with increasing concentrations of test compound or vehicle (DMSO) for 48 h in presence of 10% FCS. Cell viability was assessed according to the distributor's protocol using a microplate reader (infinite M200, Tecan Group Ltd., Crailsheim, Germany) and was calculated as a ratio of the absorbance values measured after 48 h (between test compound and vehicle (DMSO)). All experiments were tested three times and the mean ± S.E. were calculated.

2.13. Lactate dehydrogenase (LDH) cytotoxicity assay

The LDH assay (cytotoxicity detection kit; Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) was used to determine cell death after treatment of U937 cells with test compounds. LDH leakage was measured as an index of loss of cell membrane integrity. U937 cells were seeded in 96-well plates at a density of 1.5×10^4 cells/well and incubated with increasing concentrations of test compounds or vehicle (DMSO) for 48 h. Plates were centrifuged (250 × *g*, 4 min) and an aliquot of the supernatant was transferred to a clean microplate. Cell toxicity was assessed according to the distributor's protocol using a microplate reader (infinite M200, Tecan Group Ltd., Crailsheim, Germany). A control detergent supplied by Sigma–Aldrich (Saint Louis, MO, USA) was used for maximum LDH release and set to 100%. All experiments were tested three times and the mean ± S.E. were calculated.

2.14. Trypan blue exclusion

For investigation of effects on cell viability of test compounds in U937 and RPE cells, 1.6×10^5 cells/well were seeded in 6-well plates and treated with increasing concentrations of test compounds or vehicle (DMSO) for 24 h, 36 h or 48 h in the presence of 10% FCS. Afterwards, cell viability was measured by trypan blue exclusion by a cell counter (TC10™ Automated cell counter, Bio-Rad, Munich, Germany). For investigation of cytotoxic effects during activity assays, 5×10^6 freshly isolated PMNL were re-suspended in 1 ml of PGC buffer and incubated with the test compounds or vehicle (DMSO) at the indicated concentrations for 30 min at 37 °C. Afterwards, cell viability was measured by trypan blue exclusion.

2.15. Poly ADP-ribose polymerase (PARP) cleavage

U937 cells treated with test compounds for 24 h in medium containing 10% FCS or untreated control cells were centrifuged (1000 × *g*, 5 min). The cell pellet was washed once with ice-cold PBS. For the detection of PARP, cells were re-suspended in sonification buffer (PBS pH 7.4, protease inhibitor cocktail tablets (Complete, Mini; Roche diagnostics GmbH)), and disrupted by sonification (3 × 10 s, 4 °C). The resulting cell homogenates were centrifuged (10 000 × *g*, 10 min, 4 °C). Protein concentrations in the supernatant were determined using the Bradford method. Equal quantities of protein extracts were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), proteins were electrophoretically blotted onto a nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences Ltd., Little Chalfont, UK), and immunodetection of PARP was performed as described before [51]. Western blot analysis experiments were performed in triplicate.

2.16. Salmonella mutagenicity test

The Salmonella mutagenicity test for detecting carcinogenic and mutagenic potential was performed with the histidine auxotroph (his^-) *Salmonella typhimurium* strains TA98 and TA100 in assays conducted with and without metabolic activation (addition of S9 liver homogenate mix) as described by Maron and Ames [52].

2.17. Molecular modeling of the putative binding mode of EP6

For molecular modeling and docking calculations, the crystal structure of engineered human 5-LO (Protein Data Bank (PDB, http://pdb.org [9]), ID: 3o8y [10], chain B) was first subjected to *in silico* mutagenesis to restore the WT enzyme. For enabling crystallization and structure determination, Gilbert et al. mutated or deleted several amino acids to obtain the so called "stable 5-LO" [10]. They introduced the following mutations: W13E, F14H, W75G, L76S, C240A, C561A, $K^{653}KK^{655} \rightarrow$ ENL, and ΔP40 to D44GS. To restore the WT enzyme, we performed *in silico* mutagenesis of the modified residues and reconstruction of the missing segment using the software package MOE (version 2010.10; Chemical Computing

Group, Montreal, Canada), which uses rotamer libraries, loop prediction [53], and energy minimization to construct the missing or modified residues. Energy minimization was performed using the all-atom force field AMBER99 [54] with RMS gradient of 1. Docking calculations were performed with PLANTS (version 1.1; University of Konstanz, Germany) [55] with the scoring function chemplp with default settings and flexible amino acid side chains (supplemental information). For the docking study a set of 200 binding poses was generated. For analysis of the docking pose, so called Protein Ligand Interaction Fingerprints (PLIF) provided by the software package MOE (version 2010.10; Chemical Computing Group, Montreal, Canada) were used. PLIFs describe a receptor–ligand complex regarding the interaction between the involved amino acid residues and a bound ligand in a bit-fingerprint scheme. The fingerprint consists of six different interaction types for each residue: side chain hydrogen-bond donor and acceptor, backbone hydrogen-bond donor and acceptor, ionic interactions and surface interactions. Additionally, each type is partitioned into weak and strong interactions, described by two separate bits. For each residue only the strongest interaction between itself and the ligand is considered and regarding the interaction type no bits (0 0) for the absence of the individual interaction, the first bit for a weak (0 1) or both bits for a strong interaction (1 1) will be set. The probability of a good hydrogen-bond interaction is based on the distance and orientation of the considered atom pair regarding protein contact statistics. The ionic interactions are scored by the inverse square of the distance between two opposite formal charged atoms. The surface contact interactions are described by the difference of the solvent exposed surface area of the residues in the unbound and bound protein–ligand complex.

2.18. Statistics

Data are expressed as mean values with S.E. All IC_{50} values are means with S.E. of the IC_{50} values obtained from measurements at three to five different concentrations of the compounds in three to five independent experiments. Each IC_{50} value of an independent experiment is an approximation determined by graphical analysis (linear interpolation between the points at 50% activity) using SigmaPlot2006 (version 10.0, Systat Software Inc., Chicago, USA). Data were subjected to one- or two-way ANOVA with Tukey *post hoc* test, classification of P according to: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

3. Results

3.1. EP6 inhibits 5-LO in intact cells and cell-free systems

The inhibitory effect of **EP6** on 5-LO product formation was determined in different 5-LO preparations. **EP6** inhibited 5-LO in calcium ionophore stimulated intact PMNL, homogenates of PMNL and S100 preparations of the homogenates with comparable IC_{50} values (Fig. 2, no significant differences among the IC_{50} values). In intact cells and in homogenates, **EP6** exhibited an IC_{50} value of 0.16 μM (±0.01 μM) and 0.21 μM (±0.03 μM). The IC_{50} values for inhibition of S100 and partially purified 5-LO were 0.11 μM (±0.01 μM) and 0.05 μM (±0.01 μM), respectively. **EP6** inhibited purified 5-LO with a higher potency than in intact PMNL or homogenates ($^{*}P < 0.05$ and $^{**}P < 0.01$). Therefore, we conclude that **EP6** is a direct inhibitor of 5-LO.

3.2. Investigations of influence of substrate concentration on the inhibitory potency of EP6

To investigate the type of inhibition of **EP6**, S100 samples were stimulated with increasing substrate concentrations (1–30 μM).

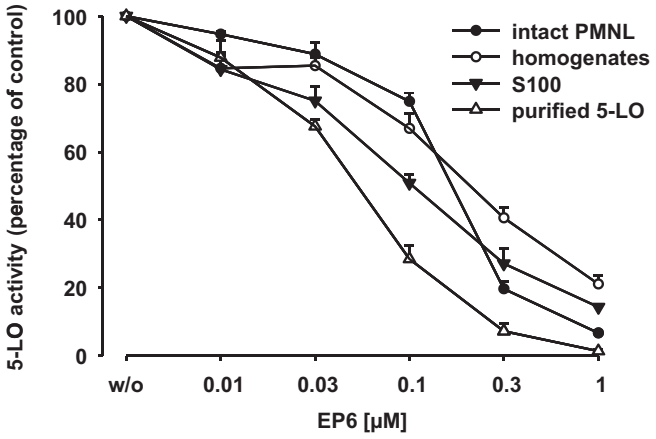

Fig. 2. Inhibition of 5-LO activity by **EP6**. 5×10^6 intact PMNL were re-suspended in PGC buffer and pre-incubated for 15 min at 37 °C with the indicated concentrations of **EP6** or vehicle (DMSO). 5-LO product formation was started by addition of 2.5 μM calcium ionophore and 20 μM AA, after 10 min 5-LO product formation was determined. For determination of 5-LO product formation in cell-free systems, homogenates and S100 preparations corresponding to 7.5×10^6 PMNL, or partially purified 5-LO were dissolved in 1 ml PBS, pH 7.4, 1 mM EDTA and 1 mM ATP and were pre-incubated for 15 min with **EP6** or vehicle (DMSO) at the indicated concentrations at 4 °C. Samples were pre-warmed at 37 °C for 30 s and 5-LO product formation was started by addition of 2 mM $CaCl_2$ and 20 μM AA. After 10 min, 5-LO product formation was determined. Data (mean ± S.E.; $n \geq 3$) are expressed as percentage of control.

AA had no direct influence on the inhibitory potency of **EP6** (Fig. 3A). Furthermore intact PMNL were stimulated via calcium ionophore A23187 without or with addition of exogenous AA (20 μM). The IC_{50} value in presence of 20 μM exogenously added AA was 0.18 μM (±0.06 μM), but in absence of exogenous substrate, an IC_{50} value of 1.14 μM (±0.32 μM) was determined (Fig. 3B). The ratio of the 5-LO products 5-H(p)ETE to LTB_4 increased in the control if exogenous AA (20 μM) was added, but the formation of the 5-LO metabolite LTB_4 and the formation of 5-H(p)ETE was thereby inhibited with a similar potency (data not shown). The FLAP antagonist MK-886 and the iron-ligand inhibitor zileuton served as controls and exhibited IC_{50} values of 0.04 μM (±0.001 μM) and 0.51 μM (±0.13 μM) without exogenously added AA, and with addition of 20 μM AA IC_{50} values of 0.03 μM (±0.003 μM) and 1.6 μM (±0.79 μM), respectively (data not shown). Moreover, zileuton did not change the ratio of 5-LO products formed, regardless the presence of exogenous AA, while MK-886 inhibited LTB_4 formation more potently than 5-H(p)ETE in presence of exogenous AA (data not shown).

3.3. Inhibition of 5-LO product formation by EP6 is stimulus-independent

The activation of 5-LO in intact cells is modulated via increase of intracellular calcium leading to translocation of the enzyme from the cytosol to the nuclear envelope [56] or by phosphorylation of the enzyme [29,30]. Moreover, the cellular redox tone regulates the enzymatic activity [57] and a certain hydroperoxide level is required for conversion of 5-LO from the ferrous to the ferric form [58]. The activity of nonredox-type inhibitors strongly depends on the mode of enzyme activation and the cellular redox tone [26]. A common *in vitro* stimulus for the evaluation of 5-LO inhibitors is the non-physiological stimulus calcium ionophore A23187 leading to cellular calcium influx. Other stimuli are cell stress such as chemical stress (sodium arsenite (SA)) or hyperosmotic stress (NaCl), which in turn activate 5-LO via phosphorylation. After stimulation of intact PMNL with 300 mM NaCl, 10 μM SA or 2.5 μM calcium ionophore A23187, the efficacy

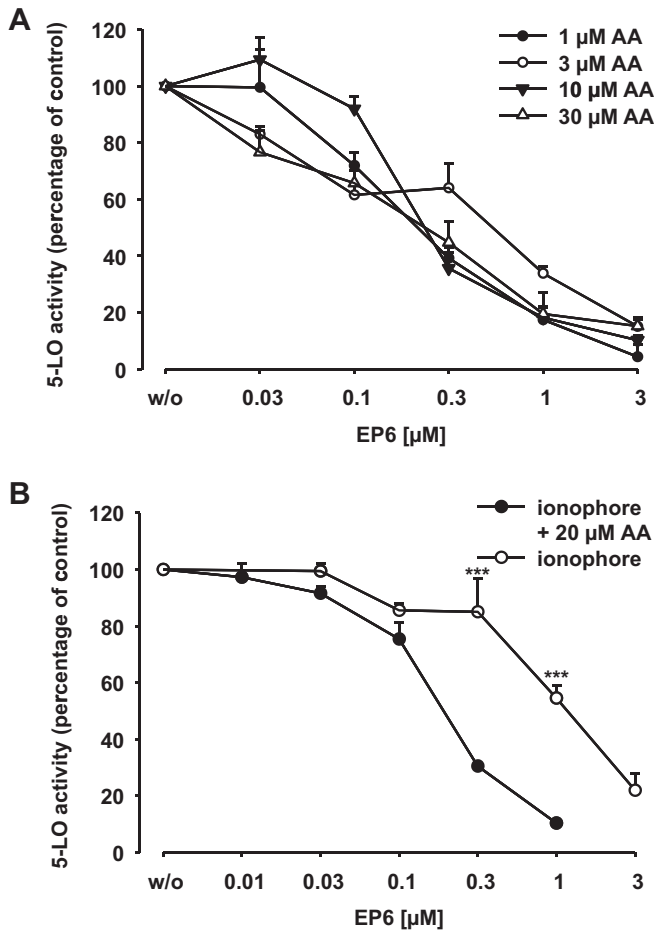

Fig. 3. Influence of substrate concentration on inhibition of 5-LO by **EP6**. (A) S100, corresponding to 7.5×10^6 PMNL were incubated as described for Fig. 2 with AA at the indicated concentrations and 5-LO product formation was determined. Data (mean ± S.E.; $n \geq 3$) are expressed as percentage of control. (B) 7.5×10^6 or 1×10^7 PMNL were incubated as described for Fig. 2 with or without 20 µM AA and 5-LO product formation was determined. Data (mean ± S.E.; $n \geq 3$) are expressed as percentage of control. $^{***}P < 0.001$ versus controls without AA addition at corresponding concentrations of test compound, two-way ANOVA + Tukey *post hoc* test.

of **EP6** was identical (Fig. 4A) and IC_{50} values of 0.2 µM were determined. **EP6** inhibits 5-LO in intact cells independently of these three stimuli for 5-LO activation. Furthermore a more physiological stimulus with LPS and fMLP was investigated. Intact PMNL were primed with 1 µg/ml LPS and 0.2 U/ml Ada and stimulated via 1 µM fMLP with or without addition of 20 µM AA (Fig. 4B). The inhibitory activity of **EP6** was reduced in intact PMNL after stimulation via LPS and fMLP compared to the other stimuli: an IC_{50} value of 0.74 µM (±0.06 µM) was determined in presence of exogenously added AA (Fig. 4B). Without exogenously added substrate the IC_{50} value even increased to 1.77 µM (±0.74 µM). Addition of exogenous AA (20 µM) led to an increase in the ratio of the 5-LO products 5-H(p)ETE to LTB_4, but both 5-LO metabolites (LTB_4 and 5-H(p)ETE) were inhibited with similar potency (data not shown). For the FLAP antagonist MK-886 IC_{50} values of 0.013 µM (±0.002 µM) in absence of exogenous AA and of 0.08 µM (±0.04 µM) in presence of exogenous AA were determined. Zileuton inhibited 5-LO product formation after stimulation via LPS and fMLP with IC_{50} values of 0.3 µM in absence of exogenous AA and of 0.66 µM (±0.07 µM) in presence of 20 µM exogenously added AA (data not shown). Again, while MK-886 inhibited LTB_4 formation more potently than 5-H(p)ETE in presence of exogenous AA, zileuton did not change the ratio of these metabolites (data not shown).

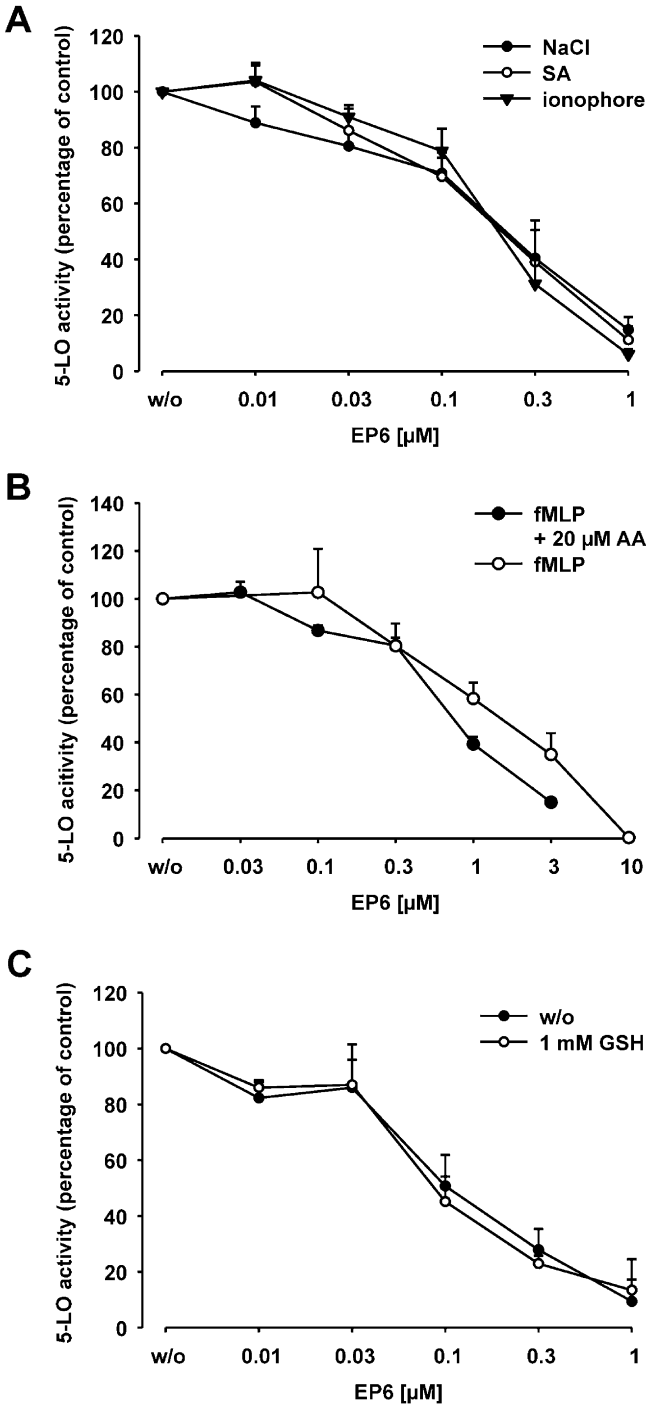

Fig. 4. Effects of different stimuli for 5-LO activation on 5-LO inhibition by **EP6**. (A) 7.5×10^6 freshly isolated PMNL were re-suspended in 1 ml PGC buffer and **EP6** or vehicle (DMSO) was added at the indicated concentrations. After 12 min at 37 °C, cells were pre-incubated with 300 mM NaCl or 10 µM SA for 3 min and 20 µM AA was added, or after 15 min at 37 °C cells were stimulated with 2.5 µM calcium ionophore A23187 together with 20 µM AA. After 10 min incubation, 5-LO product formation was determined. Data (mean ± S.E.; $n \geq 3$) are expressed as percentage of control. (B) 7.5×10^6 or 1×10^7 PMNL were primed with 1 µg/ml LPS and 0.2 U/ml Ada for 15 min at 37 °C, pre-incubated for 15 min at 37 °C with the indicated concentrations of **EP6** or vehicle (DMSO) and 5-LO product formation was stimulated by addition of 1 µM fMLP with or without addition of 20 µM AA. Data (mean ± S.E.; $n \geq 3$) are expressed as percentage of control. (C) S100, corresponding to 7.5×10^6 PMNL were incubated as described for Fig. 2 in the absence or presence of 1 mM GSH at 4 °C. Data (mean ± S.E.; $n \geq 3$) are expressed as percentage of control.

To investigate the influence of the redox tone on the potency of **EP6**, GSH, a cysteine-containing, redox-active tripeptide, was added in a cell free system. GSH serves as co-substrate of glutathione peroxidases leading to reducing conditions. The incubation of S100 samples with 1 mM GSH had no impact on the efficacy of **EP6** (Fig. 4C).

3.4. Influence of allosteric factors on the efficacy of EP6

The C2-like domain of 5-LO interacts with allosteric factors like ATP [15] and calcium [14] and mediates binding to cellular membranes [11] or phospholipid vesicles [12]. These allosteric factors are thought to induce conformational changes upon binding to 5-LO; therefore we investigated the interference of these factors with 5-LO inhibition by **EP6**. No influence on the inhibitory activity of **EP6** in cell-free systems could be measured for ATP and calcium (supplemental Fig. S1). In contrast, PC affected the inhibitory activity of **EP6**: to mimic 5-LO binding to cellular membranes, S100 of *E. coli* cultures of WT 5-LO were incubated with exogenously added PC (Fig. 5A). The presence of 20 μg/ml PC reduced the efficacy of **EP6** to an IC_{50} value of 1.04 μM (±0.28 μM). The IC_{50} value of the control, S100 of *E. coli* cultures of WT 5-LO without exogenously added PC, was 0.21 μM (±0.01 μM). In order to confirm the influence of PC on the interaction of 5-LO with **EP6**, the inhibitory potency of **EP6** in S100 of *E. coli* cultures of a 5-LO-3W mutant was investigated (Fig. 5B). Three tryptophan residues (W13, 75, 102) facilitating binding of PC [12], are mutated to alanine in this mutant. The 5-LO-3W mutant exhibits a reduced PC binding affinity thus the stimulating effect of PC is lower for the 5-LO-3W mutant in comparison to 5-LO WT [59]. For S100 of 5-LO-3W mutant, an IC_{50} value of 1.74 μM (±0.54 μM) without exogenously added PC and an IC_{50} value of 1.43 μM (±0.53 μM) with exogenously added PC was determined (Fig. 5B). **EP6** inhibited the S100 of 5-LO-3W mutant significantly less effective than the WT enzyme (Fig. 5B).

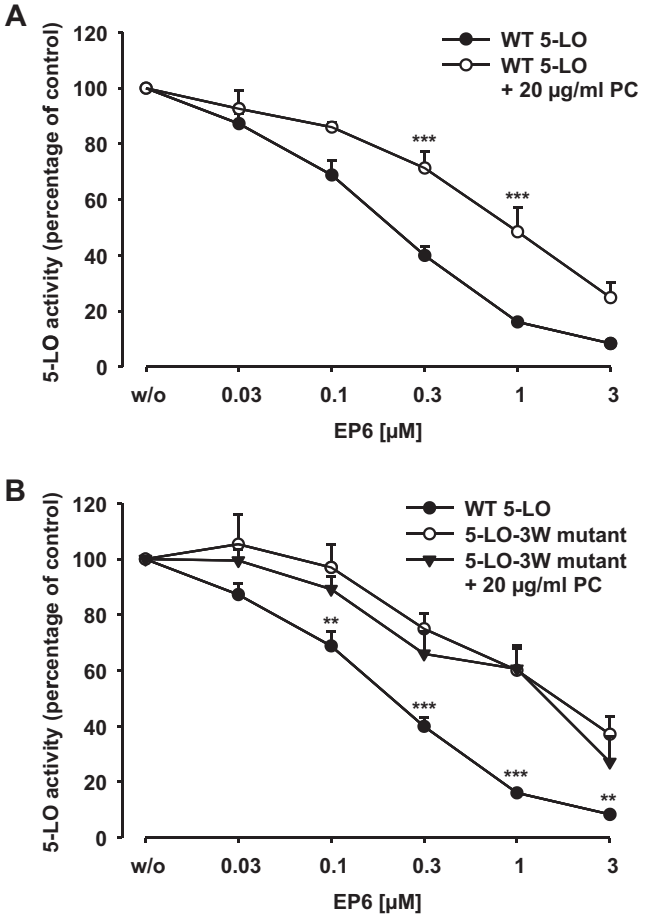

Fig. 5. Influence of allosteric factors on the inhibition of 5-LO by **EP6**. (A) S100 of WT 5-LO expressed by *E. coli* was incubated as described for Fig. 2 in the absence or presence of 20 μg/ml PC. Data (mean ± S.E.; $n \geq 3$) are expressed as percentage of control. $^{***}P < 0.001$ versus controls without PC addition at corresponding concentrations of test compound, two-way ANOVA + Tukey *post hoc* test. (B) S100 of WT 5-LO or 5-LO-3W mutant expressed by *E. coli* was incubated as described for Fig. 2. Data (mean ± S.E.; $n \geq 3$) are expressed as percentage of control. $^{**}P \leq 0.01$; $^{***}P < 0.001$ versus 5-LO-3W mutant at corresponding concentrations of test compound, two-way ANOVA + Tukey *post hoc* test.

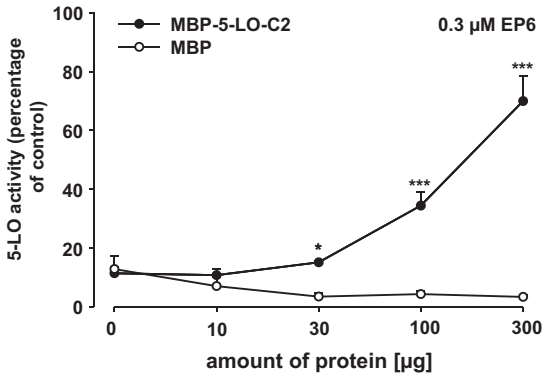

Fig. 6. C2-like domain competition assay. Increasing amounts (0–300 μg/ml) of MBP-5-LO-C2 were added to 2–3 μg partially purified 5-LO in 1 ml PBS, pH 7.4, 1 mM EDTA and 1 mM ATP. γ-Globuline was added to adjust consistent protein concentration (300 μg/ml). The samples were pre-incubated for 15 min with 0.3 μM **EP6** or vehicle (DMSO) at 4 °C. 5-LO product formation was stimulated and determined as described for Fig. 2. Data (mean ± S.E.; $n \geq 3$) are expressed as percentage of control (DMSO with the same amount of added protein). $^{*}P \leq 0.01$; $^{***}P < 0.001$ versus MBP at corresponding amounts, two-way ANOVA + Tukey *post hoc* test.

3.5. 5-LO C2-like domain: a putative binding region for EP6

To evaluate the interaction of **EP6** with the C2-like domain of 5-LO, a C2-like domain competition assay was performed. Partially purified 5-LO was incubated with 0.3 μM of **EP6** yielding a relative 5-LO activity of 13 ± 1% (Fig. 6). Increasing amounts of C2-like domain were added as fusion protein of MBP and C2-like domain (due to purification requirements). Increasing amounts of MBP alone were

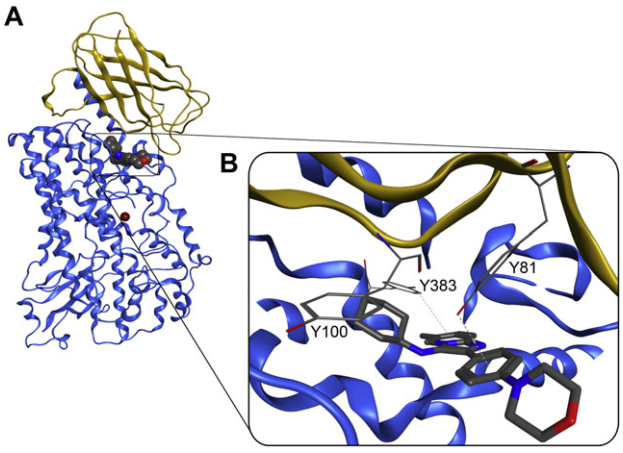

Fig. 7. (A) Structure of the restored 5-LO model of the crystal structure of engineered 5-LO [10]. Yellow: C2-like domain, blue: catalytic domain, red: active site iron, **EP6** is depicted in a spherical calotte representation. (B) Docking of **EP6** in the interface of the C2-like (yellow) and catalytic (blue) domain. Putative interacting amino acids are depicted and possible interactions are marked. **EP6** and interacting amino acids are depicted in stick representation colored by atom types.

added as control. Addition of MBP did not impair the inhibition of 5-LO by **EP6**. In contrast, the addition of MBP-5-LO-C2 led to an abolishment of the inhibition to 70 ± 8% of the relative 5-LO activity (Fig. 6). The presence of 300 μg of fusion protein of MBP and C2-like domain resulted in a low stimulation (118 ± 2% of control without MBP-C2-like domain, data not shown) of the purified 5-LO due to stabilizing scaffold properties of the C2-like domain. These results corroborate an interaction of **EP6** with the C2-like domain of 5-LO.

3.6. Docking of EP6 and mutagenesis studies for identification of a potential binding mode

For evaluation of the results of the C2-like domain competition assay, an *in silico* docking was performed for **EP6** with a restored 5-LO model of the crystal structure of engineered human 5-LO [10]. The binding pocket was defined in the interface between the catalytic and the C2-like domain of the 5-LO (Fig. 7). The docking study of **EP6** performed by a flexible side chain docking with PLANTS generated a set of 200 binding poses. The analysis of the conformations resulted in two different major orientations in which **EP6** was predicted to bind to the 5-LO.

To validate the statistical relevance of the different conformations and their underlying interactions PLIF were calculated for each pose (supplemental Fig. S2). The relative frequency of the described PLIF interactions, especially with Y81 (99.5%) and Y383 (96.5%) (supplemental Fig. S2 and Fig. 7B) is potentially indicative for the binding pose of **EP6** shown in Fig. 7B. The tyrosines are able to interact with aromatic ring systems of **EP6** via π interactions (Fig. 7B) with the imidazo[1,2-*a*]pyridine core (Y383) and the aromatic moiety neighboring the morpholine group. The morpholine group of **EP6** which is supposed to shield the bound inhibitor from the solvent, points out of the binding pocket (Fig. 7B and supplemental Fig. S3).

To verify these *in silico* results that represent a possible binding mode, two tyrosine residues within the C2-like domain (Y81, Y100) were replaced by alanine via site-directed mutagenesis. Y100 was positioned in the immediate receptor contact within the binding pocket (supplemental Fig. S3). The competition assay with the C2-like domain (*cf* Fig. 6) was repeated with a MBP-5-LO-C2 Y81/100A mutant. The addition of MBP-5-LO-C2 Y81/100A led to a much lower maximal reduction of the 5-LO inhibition (34 ± 3% remaining activity (Fig. 8)) compared to the addition of the WT C2-like domain (remaining enzyme activity: 70 ± 8% (Fig. 6)) confirming an involvement of these tyrosines in **EP6** binding. For specification of

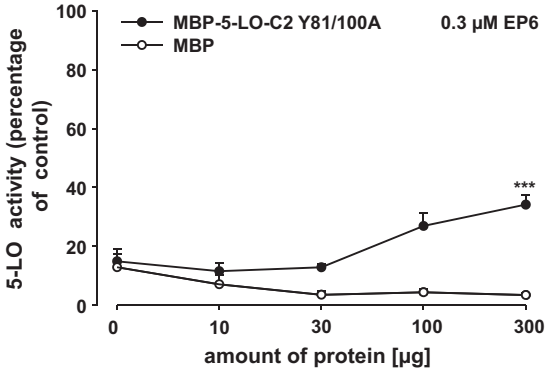

Fig. 8. C2-like domain competition assay. Increasing amounts (0–300 μg/ml) of MBP-5-LO-C2 Y81/100A were added to 2–3 μg partially purified 5-LO in 1 ml PBS, pH 7.4, 1 mM EDTA and 1 mM ATP. γ-Globuline was added to adjust consistent protein concentration (300 μg/ml). The samples were pre-incubated for 15 min with 0.3 μM **EP6** or vehicle (DMSO) at 4 °C. 5-LO product formation was stimulated and determined as described for Fig. 2. Data (mean ± S.E.; $n \geq 3$) are expressed as percentage of control (DMSO with the same amount of added protein). $^{***}P < 0.001$ versus MBP at corresponding amounts, two-way ANOVA + Tukey *post hoc* test.

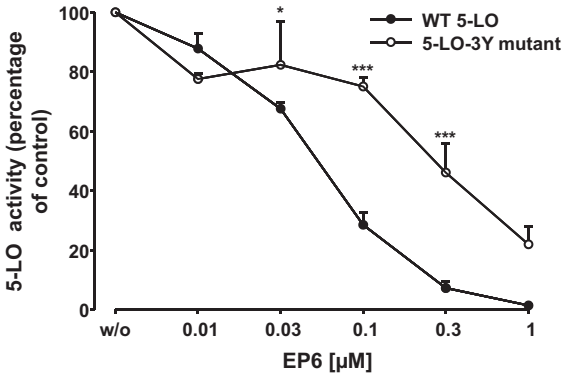

Fig. 9. Inhibition of partially purified 5-LO-3Y mutant by **EP6**. 2–3 μg partially purified 5-LO WT or 5-LO-3Y mutant were incubated as described for Fig. 2. Data (mean ± S.E.; $n \geq 3$) are expressed as percentage of control. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P < 0.001$ versus WT 5-LO at corresponding concentrations of test compound, two-way ANOVA + Tukey *post hoc* test.

this putative binding mode, the tyrosines (Y81, Y100, and Y383) were replaced by alanine within the whole enzyme. Single mutants (Y81A, Y100A and Y383A), a C2-like domain double mutant (Y81/100A) and a triple mutant (Y81/100/383A, 3Y) were investigated. The replacement of the tyrosines within the C2-like domain led to no change in the inhibitory efficacy of **EP6** (Table 1). The IC_{50} values were comparable (no significant differences) for the 5-LO WT and the three C2-like domain mutants (IC_{50} = 0.03 μM (±0.01 μM)–0.08 μM (± 0.04 μM), Table 1). However, the mutation of tyrosine at position 383 yielded an IC_{50} value of 0.14 μM (±0.02 μM) implying an interaction of this amino acid with the compound. Furthermore, **EP6** inhibited the 5-LO-3Y mutant with an IC_{50} value of 0.23 μM (±0.08 μM), significantly ($^{**}P < 0.01$) less potent than the 5-LO WT (IC_{50} = 0.05 μM (±0.01 μM)) (Fig. 9). The specific activities of 5-LO WT and the mutants are comparable (Table 1), implying native fold proteins with no impaired catalytic activity. The results of the 5-LO-3Y mutant confirmed an impact of the C2-like domain (via Y81 and Y100) and corroborate the results of the C2-like domain competition assay.

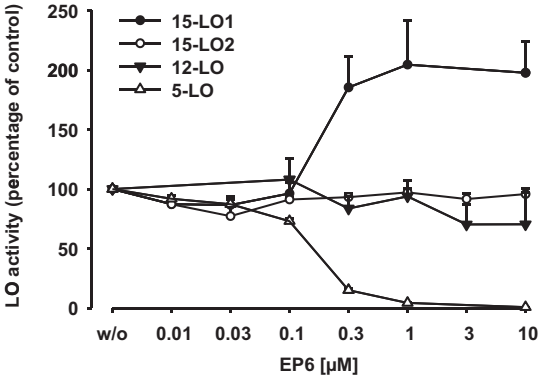

Fig. 10. Selectivity profile of **EP6**. (A) For determination of 5-, 12-LO and 15-LO1 product formation, 5×10^6 intact PMNL or 1×10^8 intact platelets were re-suspended in PGC buffer and pre-incubated for 15 min at 37 °C with the indicated concentrations of **EP6** or vehicle (DMSO). LO product formation in PMNL was started by addition of 2.5 μM calcium ionophore and 20 μM AA. For 12-LO activity in platelets 10 μM AA was added. For determination of 15-LO2 activity, 0.5 U human recombinant 15-LO2 was dissolved in 1 ml PBS, pH 7.4 and pre-incubated for 15 min on ice with the indicated concentrations of **EP6** and vehicle (DMSO). 15-LO2 activity was started by adding 100 μM AA. After 10 min, 5-, 12-LO and 15-LO product formation was determined. 12-LO products include 12(*S*)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (12-H(p)ETE). 15-LO products were 15(*S*)-hydro(pero)xy-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid (15-H(p)ETE). Data (mean ± S.E.; $n \geq 3$) are expressed as percentage of control.

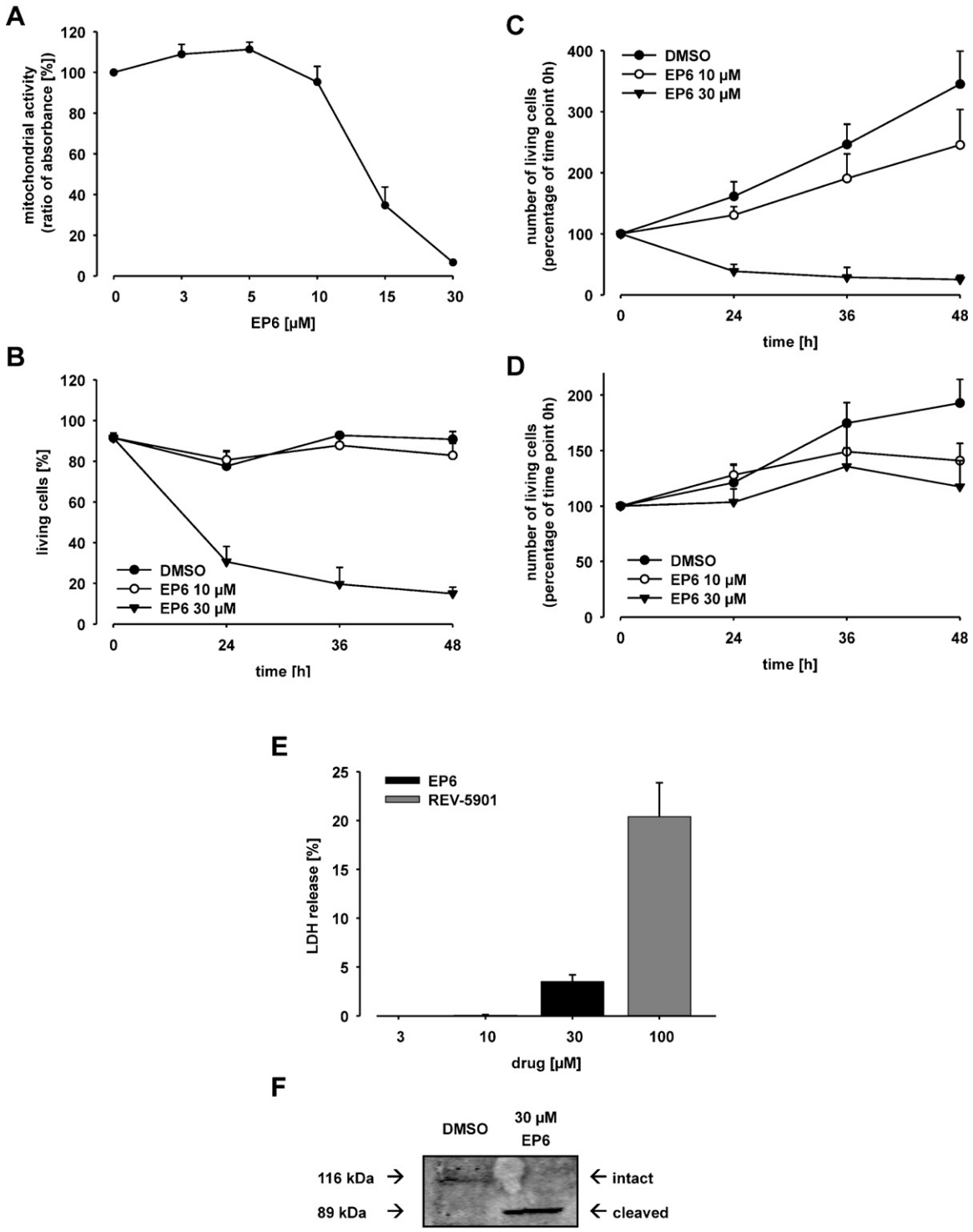

Fig. 11. Effects of **EP6** on cell viability. (A) U937 cell viability after 48 h treatment with increasing concentrations of **EP6** (3–30 μM) was assessed using a WST-1 proliferation assay. Values, measured as ratio of absorbance for a given concentration of **EP6** and the control (DMSO) after 48 h, present mean ± S.E. of three independent experiments. (B) Long-term viability assay with U937 cells. Cells were treated with 10 μM and 30 μM **EP6** for 24 h, 36 h and 48 h and the survival rates, measured as percentage of living cells compared to total cell number at a given time point, were measured by trypan blue exclusion. Values are given as mean ± S.E. of three independent experiments. (C) Long-term viability assay with U937 cells. Cells were treated with 10 μM and 30 μM **EP6** for 24 h, 36 h and 48 h and the survival rates were measured by trypan blue exclusion. The survival rates were calculated as ratio of the number of living cells at time point 0 h and a particular time point. Values are given as mean ± S.E. of three independent experiments. (D) Long-term viability assay with RPE cells. Cells were treated with 10 μM and 30 μM **EP6** for 24 h, 36 h and 48 h and the survival rates were measured by trypan blue exclusion. The survival rates were calculated as ratio of the number of living cells at time point 0 h and a particular time point. Values are given as mean ± S.E. of three independent experiments. (E) Effect of **EP6** on LDH release by U937 cells. Cells were incubated with increasing concentrations (3–30 μM) of **EP6** or 5-LO inhibitor Rev-5901 (100 μM) for 48 h. LDH release induced by a control detergent supplied by Sigma–Aldrich (Saint Louis, MO, USA) was set to 100%. Values are given as mean ± S.E. of three independent experiments. (F) Western blot analysis of PARP cleavage after treatment of U937 cells with 30 μM **EP6** for 24 h. 35 μg of total protein extract was separated on a 10% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane. Cleaved and intact PARP were detected using a specific antibody. One of three independent experiments is shown.

Table 1
IC_{50} values for inhibition of partially purified WT 5-LO and 5-LO-Y mutants. Mean of three experiments.

Mutant	IC_{50} value [μM]	Specific activity (ng products/μg protein)
WT	0.05 (±0.01)	361 (±151)
Y81A	0.06 (±0.02)	398 (±61)
Y100A	0.08 (±0.04)	308 (±70)
Y383A	0.14 (±0.02)	202 (±4)
Y81/100A	0.03 (±0.01)	262 (±32)
3Y	0.32 (±0.08)	260 (±85)

3.7. Investigations on pleiotropic effects and influence on cell viability of EP6

Furthermore, pleiotropic effects of **EP6** regarding several other AA binding proteins were investigated. In intact PMNL 5-LO selectivity was determined. **EP6** did not inhibit 15-LO1 (Fig. 10). The increase in 15-LO1 products could be due to the shunt of substrate to 15-LO1 when 5-LO is inhibited at least in our experimental setting where exogenous AA is present. Recombinant human 15-LO2 was not inhibited by **EP6** either (Fig. 10). In addition, the effect on platelet-type 12-LO was investigated (Fig. 10). No reduction of 12-H(p)ETE was measured. Three additional enzymes of the AA cascade, COX-1, COX-2 and mPGES-1, were investigated. COX-1 and COX-2 inhibition up to a concentration of 10 μM of **EP6** was not observed. Within the COX-1 assay, 10 μM **EP6** exhibited a residual activity of 93.6 ± 6% of control, 1 μM of the COX-1 inhibitor SC-560 [60] led to 4.4 ± 1% of control. For COX-2 residual activities of 101 ± 7% of control for 10 μM **EP6** and 21.8 ± 6% of control for 1 μM of the COX-2 inhibitor rofecoxib [61] were measured (data not illustrated). **EP6** showed no inhibitory effect on mPGES-1 activity in a cell-free assay up to 30 μM as well. For 30 μM **EP6** a residual activity of 89.4 ± 19% of control was determined (data not illustrated). For the known mPGES-1 inhibitor MD52 [62] a residual activity of 32.2 ± 13% at 0.3 μM was measured.

A salmonella mutagenicity test [52] for detecting a carcinogenic and mutagenic potential of **EP6** led to neither frameshift nor substitution mutations independent of oxidative metabolism up to 100 μM **EP6** (data not shown).

We further tested **EP6** in a murine leukemic monocyte macrophage cell line (supplemental Fig. S4). In intact RAW 264.7 cells an IC_{50} value of 2.1 μM (±0.45 μM) was determined. For cell-free S100 preparations of RAW 264.7 cells, 5-LO product formation was inhibited with an IC_{50} value of 0.36 μM (±0.07 μM).

Short-term viability in intact PMNL after incubation for 30 min with **EP6** at 37 °C, confirmed no cytotoxic effects during activity assays for concentrations up to 30 μM (supplemental Fig. S5). In contrast, when U937 mitochondrial metabolic activity was assessed after treatment with increasing concentrations of **EP6** using a WST-1 assay, a reduction in cellular viability could be seen. **EP6** reduced the number of viable cells by 50% at a concentration of 14 μM (Fig. 11A) after 48 h. The WST-1 assay does not distinguish between anti-proliferative or cytotoxic effects. Impaired cell viability in the WST-1 assay may be due to a reduction of the total number of viable cells caused by anti-proliferative properties of **EP6**. Alternatively, **EP6** may possess cytotoxic properties inducing necrosis or apoptosis. Therefore, we investigated the influence on cell viability of U937 and non-tumor RPE cells after treatment with 30 μM **EP6** for 24 h, 36 h and 48 h by trypan blue exclusion. The survival rate for U937 cells after treatment for 24 h with 30 μM **EP6** was 31 ± 8% and decreased to 15 ± 3% after 48 h (Fig. 11B) confirming the results seen in the WST-1 assay. Comparison of the number of living cells at particular time points to time point 0 h revealed a strong influence of 30 μM **EP6** on cell viability (Fig. 11C) but excluded exclusive anti-proliferative effects since the total number of living cells further decreased compared to the cell number seeded. Interestingly, **EP6** had no influence on the cell viability of the non-tumor RPE cells (overall survival rate: 94 ± 2% (data not shown)), although a slight interference with cell proliferation in these cells could be observed as the total number of viable cells did not significantly increase after treatment with the inhibitor compared to the control (Fig. 11D). To clarify whether the reduction of cellular viability in U937 resulted from cell death events like necrosis or apoptosis, we additionally investigated the loss of cell membrane integrity and PARP cleavage in **EP6** treated U937 cells. Lactate dehydrogenase (LDH) leakage is a measure for impaired membrane integrity which points to necrotic events. When treated with increasing concentrations of **EP6** up to 30 μM for 48 h, U937 cells displayed only weak LDH release (Fig. 11E). The known nonredox-type 5-LO inhibitor Rev-5901 was used as cytotoxic control [51]. Since necrotic effects could be excluded as a cause for **EP6** mediated cytotoxicity, PARP-cleavage was assessed as a marker for apoptosis. In this late apoptotic event activated caspase-3 and caspase-6 cleave the 116 kDa nuclear protein PARP specifically into a 89 kDa and 29 kDa fragment which can be detected by Western blotting. The treatment of U937 cells with 30 μM **EP6** for 24 h led to the 89 kDa cleavage product, which could not be detected in the untreated control (Fig. 11F).

4. Discussion and conclusions

In the present study we investigated the pharmacological profile of **EP6** (cyclohexyl-[6-methyl-2-(4-morpholin-4-yl-phenyl)-imidazo[1,2-*a*]pyridin-3-yl]-amine), a novel 5-LO inhibitor with distinct properties compared to compounds belonging to known classes of 5-LO inhibitors. **EP6** potently suppressed 5-LO product formation in several *in vitro* assay systems with an IC_{50} value of 0.16 μM in intact PMNL and 0.05 μM for partially purified enzyme (Fig. 2). This direct inhibitor exhibited selectivity for 5-LO and did not inhibit 12-LO, 15-LO1 and 15-LO2 (Fig. 10) or additional enzymes of the AA cascade, COX-1, COX-2 and mPGES-1. **EP6** was active in mouse leukemic monocyte macrophage cells and suppressed 5-LO in intact and cell-free assays (supplemental Fig. S4). In addition, the compound showed no mutagenic potential and did not impair the viability of normal human RPE cells up to a concentration of 30 μM, prerequisites for future in vivo investigations (Fig. 11D).

In contrast to this, impaired cell viability of human leukemic monocytic cells (U937) (Fig. 11) with overall viability being reduced by 50% at 14 μM in a WST-1 assay (Fig. 11A) and decreased cell survival (15 ± 3%) in a trypan blue exclusion assay (Fig. 11B) was seen after treatment with 30 μM of **EP6** for 48 h. Cell counts in the trypan blue exclusion assay decreased in comparison to seeded cell numbers excluding an anti-proliferative mechanism as exclusive reason for the effect on cell viability of **EP6**. LDH release was only weakly triggered in higher concentrations (3.5 ± 0.7% at 30 μM) (Fig. 11E) excluding necrosis as primary cause as well. Finally, cleavage of PARP as a marker for late apoptosis in **EP6** (30 μM) treated U937 gave a first hint to the underlying cytotoxic mechanism (Fig. 11F) which has to be investigated further in the future.

Experimental data revealed that 5-LO binds allosteric regulatory factors. These regulatory properties are mediated by the C2-like domain involving defined amino acid residues or regions within this domain. For evaluation of the binding mode and potential binding sites, the influence of these allosteric factors on the efficacy of **EP6** was tested (Fig. 5 and supplemental Fig. S1). The addition of PC to S100 of *E. coli* cultures of WT 5-LO resulted in a shift of the IC_{50} value from 0.21 μM (±0.01 μM) to 1.04 μM (±0.28 μM) (Fig. 5A). Experiments with S100 of WT 5-LO or 5-LO-3W mutant expressed by *E. coli* corroborate this result: the 5-LO-3W mutant exhibits a reduced stimulating effect of PC due to its restricted

binding affinity of PC [58] and the inhibitory potency of **EP6** for this mutant is unaffected by the addition of PC (Fig. 5B). In comparison, the 5-LO-3W mutant, which in general can be inhibited by 5-LO inhibitors [59], is less suppressed by **EP6** than the WT 5-LO (Fig. 5B). The tryptophans W13, W75 and W102 which are responsible for interaction with PC, seem to be, directly or indirectly via conformational changes, involved in the interaction of **EP6** with 5-LO. The C2-like domain competition assay confirms a binding site located at the C2-like domain (Figs. 6 and 8). Furthermore three tyrosines, two within the C2-like domain (Y81, Y100) and one in the catalytic domain (Y383) (Fig. 7), were derived from an *in silico* docking and emphasized via mutagenesis studies (Figs. 6, 8 and 9) the interface of the C2-like and the catalytic domain as a possible binding region for **EP6**. Although the single mutants and double mutant did not show impaired IC_{50} values for inhibition of 5-LO (Table 1), the 5-LO-3Y mutant did exhibit a significantly lower IC_{50} value for inhibition of 5-LO compared to WT 5-LO. The interaction of Y383 and **EP6** alone seems to be strong enough for enzyme inhibition. Together, these findings indicate a new binding site for inhibitors at the C2-like domain of the enzyme. Allosteric regulation of LOs is known [63,64] and for 5-LO, allosteric inhibitor binding could be shown for two compounds: Acetyl-11-keto-β-boswellic acid [65] and hyperforin [66] were identified to bind to the C2-like domain of 5-LO [59,67]. Targeting this allosteric site might open up a new promising approach for 5-LO inhibition.

Compound **1** [39] was assigned by Pergola and Werz to a new group of diverse 5-LO inhibitors [68]. Redox-active inhibitors with reducing properties, iron ligand inhibitors chelating the active site iron and nonredox-type inhibitors have distinct properties from **EP6** [69]. Nonredox-type inhibitors were designed as active site-directed AA mimetics [18]. A second (regulatory) fatty acid binding site [67,70] can be assumed to be located at the C2-like domain as shown for 12/15-LO [71]. Nonredox-type inhibitors like CJ-13,610 interfere with this regulatory- and with the substrate-binding cleft at the active site [72]. Characterization of **EP6** revealed no effect on the inhibitory potency by different AA concentrations (Fig. 3A). Under chronic inflammatory processes that are associated with elevated levels of lipid hydroperoxide and free AA, nonredox-type inhibitors have a reduced efficacy [26]. **EP6** might avoid this disadvantage due to its irrespective inhibitory effect in the presence of elevated AA concentrations (Fig. 3A). **EP6** indicates properties of uncompetitive inhibitors (Figs. 3B and 4B) exhibiting higher IC_{50} values in the absence of exogenous added substrate after stimulation via calcium ionophore or the more physiological stimulus fMLP. Possibly, a certain amount of AA positively modulates 5-LO activity at the second, stimulatory AA binding site [67,70] and this might be efficiently blocked by **EP6**. On the other hand, **EP6** might inhibit cytosolic 5-LO more potently than membrane-bound 5-LO: (1) in absence of exogenous AA, PMNL produce primarily membrane-mediated LTs and (2) cytosolic 5-LO needs calcium for full activation, but fMLP stimulation only causes a 4-fold lower calcium influx compared to ionophore stimulation [73]. This might explain the higher IC_{50} value for fMLP stimulation even in presence of AA and for ionophore stimulation in absence of exogenous AA. **EP6** seems to have a decreased potency at membrane-bound 5-LO. This is also in line with the observed lower IC_{50} value in presence of PC (Fig. 5A). However, we could not detect differences in the IC_{50} values for inhibition of LTB_4 and 5-H(p)ETE formation (data not shown). The same pattern was observed for zileuton, but not for the FLAP inhibitor MK-886, which inhibited LTB_4 formation more potently than 5-H(p)ETE (in presence of AA).

Another property of nonredox-type inhibitors is an impaired inhibition of purified 5-LO or 5-LO in broken cell preparations due to a dependence on a low lipid hydroperoxide tone or the presence of reducing agents like GSH or DTT [26,72]. Notably, we were able to suspend some apparent drawbacks of these inhibitors as **EP6** suppresses 5-LO independently of the addition of thiols (Fig. 4C) and has a comparable activity in intact cells and broken cell preparations (Fig. 2).

In the cellular context, 5-LO is soluble and located in the cytosol and translocates to the nuclear envelope after activation [28]. External stimuli promote an increase of intracellular calcium or phosphorylation of 5-LO by kinases of the MAPK family [29,30]. The potency of some nonredox-type inhibitors, e.g. ZM230487 and L-739.010 [22], depends on the mode of activation of the enzyme. A 10–100-fold higher inhibitor concentration is required to suppress 5-LO activity activated by phosphorylation comparable to 5-LO activated via calcium influx [27]. We were able to show that **EP6** acts independently of the mode of 5-LO activation in the cell (Fig. 4A). In this regard, the efficacy of **EP6** in vivo might be promising related to potential activity in chronic inflammatory processes associated with an increased phosphorylation status in cells [31,32]. After activation by the pathophysiologically relevant stimulus via LPS/fMLP, **EP6** inhibits 5-LO product formation although with a slightly higher IC_{50} value (Fig. 4B).

Compared to zileuton, the only 5-LO inhibitor in clinical use which inhibits 5-LO activity in various assay systems with IC_{50} values of 0.5–1 μM [8], **EP6** is up to 10-fold more potent. Furthermore, with **EP6**s molecular pharmacological profile distinct of FLAP inhibitors, which are being studied in clinical trials and benefits in respiratory diseases [74] could be demonstrated for, **EP6** might open up a novel approach for inhibition of LT formation via the hypothetical mode of action at an allosteric site of 5-LO. Besides the indication of inhibitors of LT biosynthesis in asthma, these inhibitors may have therapeutic potential in treatment of cardiovascular disease and cancer [3]. 5-LO inhibitors may be applied in these fields since the LT pathway enzymes and receptors are abundantly expressed in cancer tissues compared to healthy tissues [75]. But the underlying mechanisms through which LTs influence carcinogenesis are still incompletely understood. Effects of **EP6** on 5-LO overexpressing tumor cells were not investigated herein. However, the selectivity for 5-LO over 15-LO suggests **EP6** as a possible anticancer agent as 15-LO is known to have tumor suppressive effects [76,77]. Moreover, the tested U937 cells lack 5-LO expression and the concentrations needed to induce cytotoxic effects (50% reduction of overall viability at 14 μM) in these cells exceeded the IC_{50} value in intact PMNL (0.16 μM (±0.01 μM)) about 90-fold. Therefore, the reduction of cell viability in these cells seems to be independent of inhibition of 5-LO product formation. This has been published for a number of other 5-LO inhibitors before by our group [51] and is subject of further investigations at the moment. Interestingly, drugs based on a scaffold of N-fused imidazoles were recently recognized as novel anticancer agents with potential topoisomerase IIα inhibitory properties [78]. The promising effects of **EP6** on cell viability in 5-LO negative tumor cells and its selectivity profile, encourages further investigation of this novel 5-LO inhibitor's possible 5-LO dependent and independent anticancer properties.

Taken together, this study characterizes **EP6**, a novel direct 5-LO inhibitor exhibiting auspicious properties in various *in vitro* assays in contrast to properties of some nonredox-type inhibitors. Targeting 5-LO independently of the cellular redox tone, the substrate concentration and furthermore independent of the stimulus of 5-LO activation, **EP6** obviously possesses advantages over known 5-LO inhibitors. Our results indicate an interaction of **EP6** with the regulatory C2-like domain of the enzyme, providing a new feasible binding site for the development of novel compounds. Nevertheless, although the C2-like domain was derived as a target region, a precise binding site has not been elucidated yet. Knowledge of the precise binding mode would open up new options for structure-based optimization and development of novel compounds with the beneficial pharmacological profile of **EP6**.

Supplementary material

Synthesis procedure, molecular modeling of the putative binding mode and analysis of docking results, influence of calcium and ATP on the inhibitory potency of **EP6**, inhibitory potency in RAW 264.7 cells, and a short-term viability assay are available as supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.10.012.

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