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Celecoxib inhibits 5-lipoxygenase

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ABSTRACT

Celecoxib is a selective cyclooxygenase-2 (COX-2) inhibitor used in the therapy of inflammatory and painful conditions. Various COX-2-independent pharmacological effects, such as a chemo-preventive and tumor-regressive activity have been suggested, but the respective non-COX-2 targets of celecoxib are still a matter of research. We now demonstrate that celecoxib inhibits 5-lipoxygenase (5-LO), a key enzyme in leukotriene (LT) biosynthesis. Celecoxib suppressed 5-LO product formation in ionophore A23187-activated human polymorphonuclear leukocytes ($IC_{50} \approx 8$ μM). Similarly, celecoxib inhibited LTB_4 formation in human whole blood ($IC_{50} \approx 27.3$ μM). Direct interference of 5-LO with celecoxib was visualized by inhibition of enzyme catalysis both in cell homogenates and with purified 5-LO ($IC_{50} \approx 23.4$ and 24.9 μM, respectively). Related lipoxygenases (12-LO and 15-LO) were not affected by celecoxib. Other COX-2 inhibitors (etoricoxib and rofecoxib) or unselective NSAIDs (non-steroidal anti-inflammatory drugs, diclofenac) failed to inhibit 5-LO. In rats which received celecoxib (i.p.), the blood LTB_4 levels were dose-dependently reduced with an ED_{50} value ≈35.2 mg/kg. Together, celecoxib is a direct inhibitor of 5-LO *in vitro* and *in vivo*. These findings provide a potential molecular basis for some of the described COX-2-independent pharmacological effects of celecoxib.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are used to treat inflammation and pain. Traditional NSAIDs inhibit both isoforms of cyclooxygenases (COX), the key enzymes in the conversion of arachidonic acid (AA) to prostaglandins (PGs). The housekeeping enzyme COX-1 primarily maintains homeostasis, whereas COX-2 functions as an induced response to

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Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; cPLA$_2$-alpha, cytosolic phospholipase A$_2$-alpha; FAP, familial adenomatous polyposis; FLAP, 5-lipoxygenase-activating protein; 5-HETE, 5(*S*)-hydroxy-8,11,14-*cis*-6-*trans*-eicosatetraenoic acid; 12-HETE, 12(*S*)-hydroxy-5,8-*cis*-10-*trans*-14-*cis*-eicosatetraenoic acid; 15-HETE, 15(*S*)-hydroxy-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid; LC–MS/MS, liquid chromatography coupled with tandem mass spectrometry; 5-LO, 5-lipoxygenase; LT, leukotriene; NSAID, non-steroidal anti-inflammatory drug; PGE_2, prostaglandin E_2; PMNL, polymorphonuclear leukocytes.

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growth factors and cytokines at inflammation and tumor sites [1–3]. COX-2-selective drugs, such as etoricoxib, celecoxib or lumiracoxib, therefore demonstrate lower incidences of symptomatic ulcers and ulcer complications [4].

When first marketed, celecoxib was shown to significantly reduce polyp formation and polyp size in patients with familial adenomatous polyposis (FAP) [5]. In a subsequent clinical trial, rofecoxib, a COX-2-selective inhibitor structurally related to celecoxib, reduced the risk of colorectal adenomas [6]. COX-2 derived PGs therefore appear to play an important role in the development of colorectal neoplasia. Intake of high celecoxib doses, which clearly exceeded those for anti-inflammatory effects and COX-2 inhibition further increased the chemo-preventive effect. Therefore, COX-2 inhibition alone is not responsible for celecoxib's anticarcinogenicity. This hypothesis was confirmed by the COX-2-independent antiproliferative effects exerted by celecoxib, but not rofecoxib, on human colon carcinoma cells in cell culture and animal studies [7–9]. In recent years, several COX-2-independent molecular mechanisms of celecoxib were identified and were assumed to contribute to celecoxib's anticarcinogenic effects [10–13]. Direct *in vivo* evidence, however, remains to be substantiated.

Like PGs, leukotrienes (LTs) are downstream products of AA and exert pivotal biological functions as well as pathogenic effects in cancer and atherosclerosis [14]. Polymorphonuclear leukocytes (PMNL) and monocytes/macrophages are the major cells capable of synthesizing LTs due to a high 5-LO expression and activity and represent crucial components in chronic inflammatory diseases. In the first step of LT biosynthesis, AA is metabolized by 5-lipoxygenase (5-LO) to LTA_4. The unstable LTA_4 acts as a precursor for bioactive LTs, such as LTB_4 and the cysteinyl-LTs C_4, D_4, and E_4 [15]. Here we report that celecoxib inhibits LT formation *in vitro* and *in vivo* by direct interference with 5-LO activity.

2. Material and methods

2.1. Reagents used for the experiments

Celecoxib, etoricoxib and rofecoxib were synthesized by WITEGA Laboratorien Berlin-Adlershof GmbH, Germany. The identity and purity of these drugs were determined using HPLC (high performance liquid chromatography) and ^{1}H NMR as described previously [16] and were >99%. The calcium ionophore (A23187), GSH (glutathione), arachidonic acid, BWA4C, zileuton, MK-886 and diclofenac were purchased from Sigma–Aldrich (Munich, Germany).

2.2. Isolation of human polymorphonuclear leukocytes from venous blood

Human PMNL were freshly isolated from leukocyte concentrates obtained from St. Markus Hospital (Frankfurt, Germany). In brief, venous blood was taken from healthy adult donors and centrifuged at 4000 × *g*/20 min/RT for preparation of leukocyte concentrates. PMNL were promptly isolated by dextran sedimentation, centrifugation on Nycoprep cushions (PAA Laboratories, Linz, Austria), and hypotonic lysis of erythrocytes as described previously [17]. PMNL (7.5×10^6 cells ml^{-1}; purity > 96–97%) were finally resuspended in phosphate-buffered saline (PBS) pH 7.4 plus 1 mg ml^{-1} glucose and 1 mM $CaCl_2$ (PGC buffer).

2.3. Transfection of human HeLa cervix carcinoma cells

Human HeLa cells (Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany) were cultured in DMEM, supplemented with 10% fetal calf serum and 100 μg ml^{-1} streptomycin and 100 U ml^{-1} penicillin at 37 °C and 5% CO_2. Plasmids (pcDNA3.1-5-LO, 25 μg/1.45×10^7 cells; psg5-FLAP, 10 μg/1.45×10^7 cells) were transiently transfected into HeLa cells using the calcium phosphate method, cultured for 48 h, and assayed for 5-LO product formation as described below.

2.4. Expression and purification of 5-LO from Escherichia coli

5-LO protein was expressed in *E. coli* JM 109 cells, transformed with pT3-5-LO, and purification of 5-LO was performed as described previously [18]. In brief, cells were lysed by incubation in 50 mM triethanolamine/HCl pH 8.0, 5 mM EDTA (ethylenediaminetetraacetic acid), soybean trypsin inhibitor (60 μg ml^{-1}), 1 mM phenylmethylsulfonyl fluoride (PMSF), and lysozyme (500 μg ml^{-1}), homogenized by sonification (3 s × 15 s), and centrifuged at 100,000 × *g* for 70 min at 4 °C. The supernatant was then applied to an ATP-agarose column (Sigma A2767, Sigma–Aldrich, Munich, Germany), and the column was eluted as described previously [19]. Partially purified 5-LO was used immediately for *in vitro* activity assays.

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

For determinations in intact cells, 7.5×10^6 freshly isolated PMNL or 2×10^6 HeLa cells were resuspended in 1 ml PGC buffer. After pre-incubation with the test compounds at 37 °C for 10 min, A23187 was added (2.5 μM for PMNL, 10 μM for HeLa cells) together with or without AA. After 10 min at 37 °C, the reaction was stopped by addition of 1 ml ice-cold methanol and 5-LO metabolites formed were extracted and analyzed by HPLC as described previously [20].

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

For the determination of 5-LO activity in cell homogenates, 7.5×10^6 freshly isolated PMNL were resuspended in PBS containing 1 mM EDTA, sonicated (3 s × 10 s) at 4 °C, and 1 mM ATP was added. For determination of the activity of recombinant 5-LO, partially purified 5-LO (0.5 μg) was added to 1 ml of a 5-LO reaction mix (PBS, pH 7.4, 1 mM EDTA, 25 μg ml^{-1} phosphatidylcholine, 1 mM ATP, and 20 μg ml^{-1} γ-globulin). In some experiments GSH (5 mM) was added to the reaction mixtures as indicated. After incubation with the test compounds for 10 min at 4 °C, samples were pre-warmed for 30 s at 37 °C and 2 mM $CaCl_2$ and 20 μM AA was added. The reaction was stopped after 10 min by the addition of 1 ml ice-cold methanol and 5-LO products formed were analyzed by HPLC as described above.

2.7. Western blot analysis of 5-LO in subcellular fractions of PMNL, and of 5-LO and FLAP (5-LO-activating protein) in transfected HeLa cells

Subcellular localization of 5-LO in PMNL by cell fractionation was investigated as described previously [21]. In brief, freshly isolated PMNL (3×10^7) in 1 ml PGC buffer were pre-incubated with celecoxib for 15 min at 37 °C. Then, 2.5 μM A23187 was added, the samples were further incubated for 10 min, and subsequently chilled on ice to stop the reaction. Nuclear and non-nuclear fractions were obtained after cell lysis by 0.1% NP-40. Aliquots of these fractions were immediately mixed with the same volume of 2 × SDS-PAGE sample loading buffer, heated for 6 min at 95 °C, and analyzed for 5-LO protein by SDS-PAGE and Western blotting using the 5-LO anti-serum 1551, AK-7 (raised in rabbit, diluted 1:25) that was kindly provided by Prof. Olof Rådmark, Stockholm, Sweden. For determination of 5-LO and FLAP expression, HeLa cells were harvested by trypsinization, sonicated (3 s × 10 s) and immunoblotting was performed as described previously [8]. The antibodies used were diluted as follows: primary antibodies raised against 5-LO 1:2500, FLAP 1:500 and β-actin 1:10000. The mouse monoclonal 5-LO antibody (clone 6a12) was self-produced in collaboration with Prof. T. Dingermann, University of Frankfurt, the polyclonal FLAP antibody (ab39535) was purchased from Abcam (Cambridge, UK) and the polyclonal β-actin antibody (sc-1616) from Santa Cruz Biotechnology (Heidelberg, Germany).

2.8. Human in vitro whole blood assay

Aliquots of freshly heparinized human blood (450 μl) obtained from healthy male and female informed volunteers were pre-incubated with the drugs or vehicle (DMSO) for 30 min at 37 °C. Formation of 5-LO products was initiated by the addition of Ca^{2+}-ionophore dissolved in 50 μl autologous plasma to obtain a final concentration of 20 μM A23187 (final DMSO concentration was <1%, final volume 0.5 ml). The reaction was terminated after 15 min by rapid cooling of the plate on ice. Then, the samples were centrifuged at 1000 × *g* and 4 °C for 15 min and 5-HETE, LTB_4, 12-HETE, 15-HETE, and PGE_2 in the plasma supernatant were analyzed using LC–MS/MS. LC–MS/MS analysis was performed on an API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Darmstadt, Germany). The mass transitions used were *m/z* 335.1 → 195.0 (LTB_4), *m/z* 319.2 → 115.0 (5-HETE), *m/z* 319.2 → 178.9 (12-HETE), *m/z* 319.2 → 219.1 (15-HETE). For internal standardization the following transitions were used: *m/z* 339.2 → 196.9 ($^2H_4$-LTB_4), *m/z* 327.2 → 116.1 ($^2H_8$-5-HETE), *m/z* 327.2 → 184.1 ($^2H_8$-12-HETE), *m/z* 327.2 → 225.9 ($^2H_8$-15-HETE). Linearity of the calibration curve was proven from 0.5 to 2500 ng ml^{-1} for each eicosanoid. Mean accuracy of the assay was 99.9 ± 3.25% for LTB_4, 99.85 ± 4.8% for 5-HETE, 100.2 ± 4.8% for 12-HETE and 99.76 ± 4.4% for 15-HETE.

2.9. Rat ex vivo whole blood assay

To 250 μl heparinized venous blood (pre-dose), obtained retrobulbarily 30 min before from anesthetized 200 g Sprague–Dawley rats, A23187, dissolved in DMSO was added to obtain a final concentration of 20 μM (final DMSO concentration was <1%). The samples were then incubated at 37 °C for 15 min using a thermo-mixer (Eppendorf, Hamburg, Germany). The reaction was stopped on ice followed by centrifugation at 1000 × *g*/10 min/4 °C. Fifteen minutes after collection of the pre-dose blood the drugs were injected intraperitoneally (5, 25, 50, and 100 mg kg^{-1} celecoxib; 2 and 30 mg kg^{-1} etoricoxib; 5 mg kg^{-1} zileuton dissolved in 100% DMSO; 1 μl DMSO/g body weight). Fifteen min after the injection the animals were anesthetized again by inhalation of isoflurane and 250 μl venous blood (after-dose) from the opposite eye was handled as described above. The plasma supernatants were stored at −70 °C until LC–MS/MS analysis of eicosanoid concentrations. The content of eicosanoids in stimulated pre-dose blood was set to 100%. The drug effect on the lipoxygenase activities was then expressed as: formation of LO products [%] = after-dose lipoxygenase product formation [ng ml^{-1}]/pre-dose lipoxygenase product formation [ng ml^{-1}] × 100. In all experiments the ethics guidelines for investigations in conscious animals were obeyed and the local Ethics Committee for Animal Research approved the experiments.

2.10. Statistics

All data are presented as mean + S.E.M. (standard error of the mean). For statistical analysis, GraphPad Prism version 4.00 (GraphPad Software, San Diego, California, USA) was used. *Ex vivo* data were subjected to the Kolmogorov–Smirnov test to confirm Gaussian distribution followed by one-way ANOVA coupled with Dunnett's post *t*-test for multiple comparisons. All other data were subjected to paired, 2-sided *t*-tests. The IC_{50} and ED_{50} values were analyzed using GraphPad Prism version 4.00 and a sigmoid curve fitting model.

3. Results

3.1. Celecoxib suppresses the formation of 5-LO products in human whole blood

To investigate the effects of COX-2-selective inhibitors on eicosanoid formation, a human whole blood assay using A23187 as stimulus was applied, followed by a highly sensitive LC–MS/MS (liquid chromatography coupled with tandem mass spectrometry) methodology capable of selectively detecting a broad spectrum of eicosanoids [22]. Celecoxib, but no other COX-2 inhibitor (up to 100 μM), concentration-dependently inhibited the formation of the 5-LO products LTB_4 and 5-HETE (5(*S*)-hydroxy-8,11,14-*cis*-6-*trans*-eicosatetraenoic acid) with IC_{50} values ≈27.3 and 40.8 μM, respectively (Fig. 1A and B). Zileuton, a well-known iron-ligand inhibitor of 5-LO [23] used as positive control, caused significant reduction of LTB_4 and 5-HETE levels at 1 μM. All COX inhibitors reduced PGE_2 formation with diclofenac and celecoxib showing the highest efficacy (Fig. 1C). Celecoxib failed to significantly suppress the levels of 12-HETE (Fig. 1D), and the formation of 15-HETE (Fig. 1E) was only slightly decreased (IC_{50} > 100 μM). Table 1 illustrates the absolute values of eicosanoids formed in the

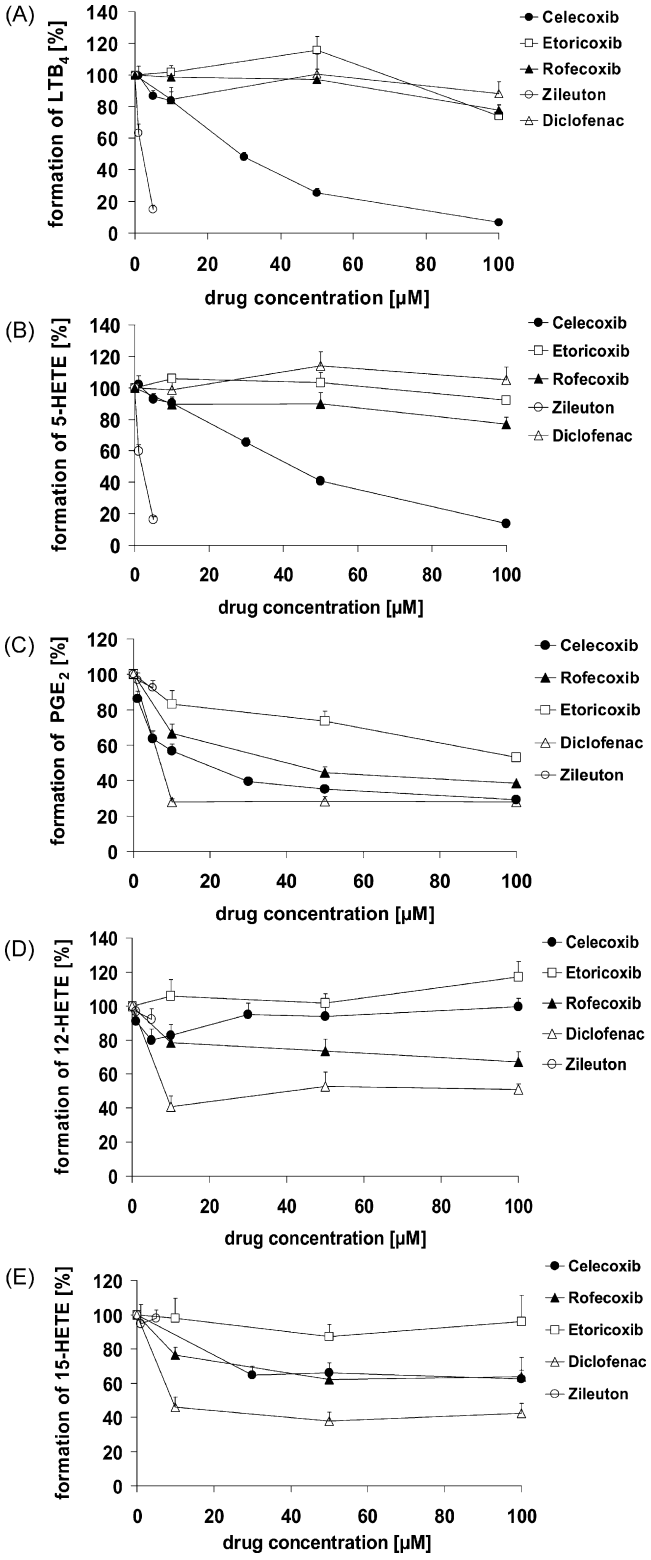

Fig. 1 – Effect of COX inhibitors on eicosanoid formation in human whole blood. The experimental procedure was performed as described in Section 2. The eicosanoids in the plasma supernatant analyzed by LC–MS/MS were LTB_4 (A), 5-HETE (B), PGE_2 (C), 12-HETE (D) and 15-HETE (E). For LTB_4, 5-HETE and 12-HETE data are given as mean + S.E.M. of 8 (celecoxib and zileuton; n = 14–26 values), 4 (etoricoxib, rofecoxib; n = 11–13 values) and 2 (diclofenac; n = 6 values) independent experiments. For PGE_2 and 15-HETE data are given as mean + S.E.M. of ≥4 (celecoxib, n = 12–15 values) or ≥2 (rofecoxib, etoricoxib, diclofenac, n = 5–6 values) independent experiments. PGE_2 formation after etoricoxib treatment was determined in a single experiment (n = 3 values).

untreated control for the whole blood assay as well as for controls in all other types of assays applied. Together, besides inhibition of PGE_2 synthesis, celecoxib has the unique property among the COX inhibitors tested to suppress the formation of 5-LO products in human whole blood.

3.2. *Celecoxib inhibits 5-LO product formation in activated human polymorphonuclear leukocytes without affecting subcellular redistribution of 5-LO*

The efficacy of celecoxib was assessed in A23187-stimulated human polymorphonuclear leukocytes, a frequently used model for evaluation of 5-LO inhibitors [24]. Pre-treatment (10 min) of PMNL with celecoxib caused a concentration-dependent inhibition of 5-LO product formation with an $IC_{50} \approx 8$ μM (Fig. 2A). To exclude effects of celecoxib on the availability of endogenous AA as substrate and thus, to circumvent $cPLA_2$-alpha activity, exogenous AA (2, 10, or 20 μM) was supplemented. However, AA supplements did not significantly alter celecoxib's inhibition of 5-LO product formation, as shown in Fig. 2A for incubations with 20 μM AA ($IC_{50} \approx 5.4$ μM), and without AA ($IC_{50} \approx 8$ μM). In agreement with the results obtained from the whole blood assay, the other coxibs etoricoxib and rofecoxib, as well as the unselective COX-inhibitor diclofenac (up to 30 μM, each), showed weak or no inhibition of 5-LO activity in A23187-activated PMNL, regardless of the presence of exogenous AA (Fig. 2B). The iron-ligand 5-LO inhibitors BWA4C and zileuton, were used as reference compounds.

5-LO is a tightly regulated enzyme and suppression of the cellular product formation by any compound does not unequivocally indicate a direct interference with 5-LO catalysis. Regulatory components or mechanisms such as FLAP, mitogen-activated protein kinases (MAPKs), Ca^{2+} mobilization, interaction with coactosin-like protein [25], and nuclear membrane translocation [26] function as indirect mediators of 5-LO activity and may also be targeted by celecoxib. Activation of 5-LO in PMNL by A23187 is accompanied by a subcellular translocation of the enzyme from the cytosol to the nuclear membrane, where 5-LO, aided by FLAP, accesses AA [27]. For some inhibitors of LT biosynthesis, such as MK-886 [26] or licofelone [28], inhibition of 5-LO translocation to the nuclear membrane was reported as one possible inhibitory mechanism. However, pre-incubation of PMNL with celecoxib caused no change in A23187-induced redistribution of 5-LO (Fig. 2C), indicating that celecoxib does not exert its suppressive effects via inhibition of 5-LO nuclear translocation. Summarized, amongst various COX inhibitors, only celecoxib suppressed 5-LO product synthesis in activated human PMNL and this effect cannot be attributed to inhibition of nuclear membrane translocation of 5-LO.

Table 1 – Absolute eicosanoid values in the untreated controls as well as respective IC_{50}/ED_{50} values for each set of experiments performed in the study

	5-HETE in untreated control	LTB_4 in untreated control	12-HETE in untreated control	15-HETE in untreated control	PGE_2 in untreated control	IC_{50} [μM] or ED_{50} [mg/kg] of celecoxib
In vitro human whole blood (LC–MS/MS)	113.34 ± 17.52 ng/ml (plasma)	73.31 ± 9.21 ng/ml (plasma)	327.06 ± 29.83 ng/ml (plasma)	14.86 ± 1.90 ng/ml (plasma)	3.00 ± 0.19 ng/ml (plasma)	40.8 (5-HETE) 27.3 (LTB_4)
PMNL intact (HPLC)						
No AA	12.14 ± 2.88 ng/10^6 cells	13.5 ± 1.59 ng/10^6 cells	n.d.	n.d.	n.d.	8.0 (5-HETE)
2 μM AA	46.3 ± 9.32 ng/10^6 cells	13.96 ± 2.34 ng/10^6 cells	n.d.	n.d.	n.d.	
10 μM AA	92.02 ± 18.14 ng/10^6 cells	16.46 ± 4.1 ng/10^6 cells	n.d.	n.d.	n.d.	
20 μM AA	93.83 ± 5.54 ng/10^6 cells	20.56 ± 1.79 ng/10^6 cells	n.d.	n.d.	n.d.	5.4 (5-HETE)
PMNL homogenates (HPLC)	46.65 ± 4.81 ng/10^6 cells	29.52 ± 8.38 ng/10^6 cells	19.44 ± 3.86 ng/10^6 cells	33.54 ± 9.5 ng/10^6 cells	n.d.	23.4 (no GSH, 5-HETE) 19.3 (+GSH, 5-HETE)
Recombinant 5-LO (HPLC)	841.5 ± 161 ng/ml	n.d.	n.d.	n.d.	n.d.	24.9 (5-HETE)
HeLa cells (HPLC)						
5-LO/psg5, 2 μM AA	2.27 ± 0.32 ng/10^6 cells	n.d.	n.d.	n.d.	n.d.	16.86 (5-HETE)
5-LO/psg5, 20 μM AA	19.38 ± 2.48 ng/10^6 cells	n.d.	n.d.	n.d.	n.d.	8.2 (5-HETE)
5-LO/FLAP, 2 μM AA	4.33 ± 0.5 ng/10^6 cells	n.d.	n.d.	n.d.	n.d.	13.07 (5-HETE)
5-LO/FLAP, 20 μM AA	24.81 ± 4.7 ng/10^6 cells	n.d.	n.d.	n.d.	n.d.	9.4 (5-HETE)
Ex vivo rat whole blood (LC–MS/MS)	56.58 ± 3.21 ng/ml (plasma)	11.95 ± 0.85 ng/ml (plasma)	13.03 ± 1.04 μg/ml (plasma)	338.13 ± 48.67 ng/ml (plasma)	n.d.	59.3 (5-HETE) 35.2 (LTB_4)

Data are mean ± S.E.M.

3.3. *Celecoxib inhibits 5-LO activity in transfected HeLa cells independently of FLAP*

Inhibition of FLAP is an alternative pharmacological strategy to suppress cellular 5-LO product synthesis. Celecoxib prevents the binding of AA to COX-2, and a similar competitive mechanism was also reported for inhibitors of FLAP [29]. Thus, it appeared possible that suppression of 5-LO product synthesis in intact PMNL by celecoxib could be due to an inhibition of the FLAP protein. To test this hypothesis, HeLa cells that express neither 5-LO nor FLAP were transiently transfected with either a 5-LO expression plasmid or with both 5-LO and FLAP expression plasmids to obtain cells that possess 5-LO only or both 5-LO and FLAP (Fig. 3A). In agreement with our previous report [18], HeLa cells expressing 5-LO and FLAP produced higher amounts of 5-LO products upon stimulation with A23187 plus 2 μM AA (about 1.9-fold) or 20 μM AA (about 1.3-fold) compared to cells transfected with 5-LO alone, supporting the functionality of FLAP in these cells. Furthermore, the FLAP-mediated increase in 5-LO product formation was abolished when the cotransfected cells were treated with the FLAP inhibitor MK-886 (1 μM), whereas MK-886 was not suppressive in HeLa cells transfected with 5-LO alone (Fig. 3B). Treatment of both cell types with celecoxib in presence of 20 μM AA caused a concentration-dependent inhibition of 5-LO product formation with an $IC_{50} \approx 8.2$ μM (5-LO-positive cells) and 9.4 μM (5-LO/FLAP-positive cells), irrespective of cellular FLAP expression (Fig. 3C). A similar reduction of 5-LO product formation in both cell types also was seen in the presence of low (2 μM) AA concentrations (Table 1), indicating that the mechanism of inhibition of celecoxib clearly contrasts with classical FLAP inhibitors. Thus, also FLAP might be excluded as relevant target for celecoxib-mediated suppression of 5-LO product formation.

3.4. *Celecoxib inhibits the activity of 5-LO in cell-free assays*

Since subcellular redistribution of 5-LO and FLAP were excluded as inhibitory mechanisms of celecoxib, direct binding of celecoxib to the 5-LO enzyme as the mechanism of suppression of product synthesis increased in likelihood. To test this hypothesis, the effect of celecoxib on 5-LO product synthesis in cell-free assays was investigated. Celecoxib concentration-dependently inhibited 5-LO product formation in homogenates of PMNL supplemented with 20 μM AA, though less potently ($IC_{50} \approx 23.4$ μM, Fig. 4A) than in intact cells ($IC_{50} \approx 8.0$ μM, Fig. 1A). Such a loss of efficacy in cell-free assays previously has been observed for nonredox-type 5-LO inhibitors [30] where addition of thiols (to reduce the lipid hydroperoxide levels via glutathione (GSH) peroxidases) restored potent inhibition. However, addition of GSH had no significant effect on the

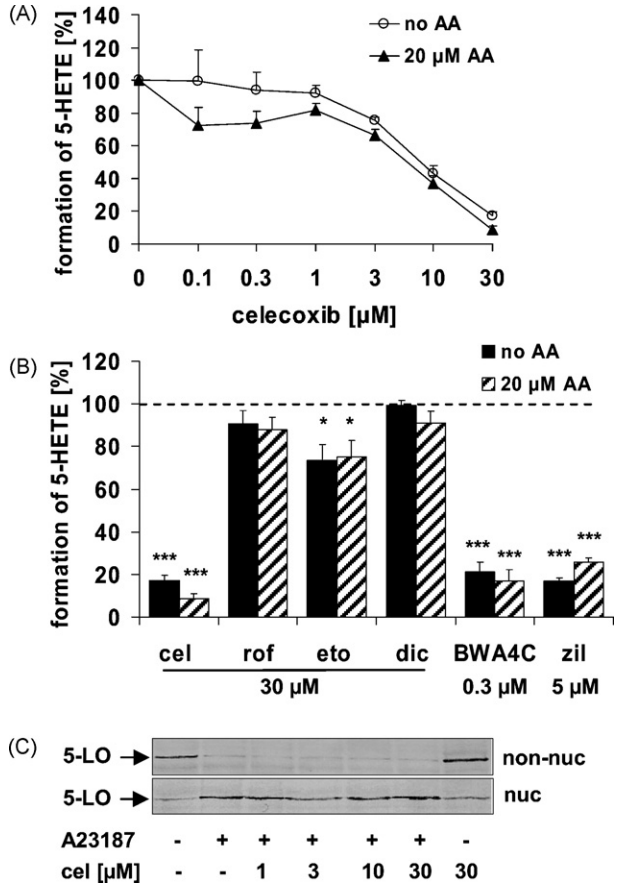

Fig. 2 – (A) Effect of celecoxib on 5-LO product formation and 5-LO subcellular redistribution in intact PMNL. 5-LO activity in absence or presence of AA (20 μM) was assayed as described in Section 2 and expressed as a percentage versus vehicle control (100%). Results are mean + S.E.M., $n \geq 7$. (B) 5-LO-inhibitory activity of celecoxib, rofecoxib, etoricoxib, diclofenac, zileuton and BWA4C in intact PMNL in the absence (black bars) or presence (striped bars) of 20 μM AA compared to vehicle control (100%). Results are mean + S.E.M., $n \geq 3$. $^{***}p \leq 0.001$; $^{*}p \leq 0.05$. (C) Effect of celecoxib on the A23187-induced subcellular redistribution of 5-LO in intact PMNL. Cells were pre-incubated with celecoxib for 15 min at 37 °C, A23187 (2.5 μM) was added and nuclear and non-nuclear fractions were analyzed for 5-LO expression using the Western blot method as described in Section 2. AA, arachidonic acid; cel, celecoxib; dic, diclofenac; eto, etoricoxib; non-nuc, non-nuclear fraction; nuc, nuclear fraction; rof, rofecoxib; zil, zileuton.

efficacy of celecoxib (Fig. 4A, $IC_{50} \approx 19.3$ μM), implying that celecoxib does not share the redox state-dependency of nonredox-type 5-LO inhibitors. In agreement with the data from whole blood assays, celecoxib (up to 30 μM) did not suppress the formation of 12-HETE or 15-HETE in homogenates of human platelets or eosinophils, respectively (Fig. 4B). Finally, celecoxib concentration-dependently inhibited the activity of partially (ATP-affinity) purified human recombinant 5-LO with an $IC_{50} \approx 24.9$ μM (Fig. 4C). Rofecoxib, etoricoxib and diclofenac (up to 100 μM), caused no or only modest inhibition of 5-LO activity in the cell-free assays (Fig. 4D and E). Celecoxib is therefore a direct inhibitor of 5-LO, a property that is unique within the family of coxib molecules.

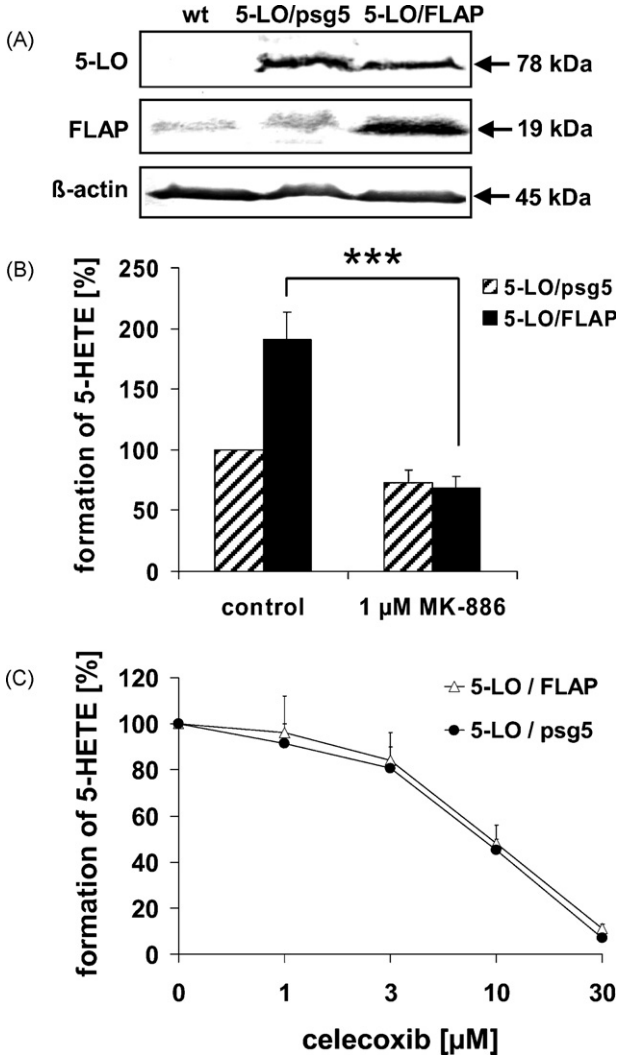

Fig. 3 – Celecoxib inhibits 5-LO product synthesis independently of FLAP. (A) Western blot analysis of 5-LO and FLAP in wild type (wt), 5-LO-transfected (5-LO/psg5) and 5-LO/FLAP-cotransfected (5-LO/FLAP) HeLa cervix carcinoma cells. Immunoblotting was performed as described in Section 2. (B) 5-HETE production in 5-LO/psg5- and 5-LO/FLAP-transfected HeLa cells after stimulation with 10 μM A23187 in presence of 2 μM AA (control) and effect of 1 μM MK-886 on 5-LO activity in both cell types. 5-LO activity was determined as described in Section 2 and expressed as percentage compared to 5-LO/psg5-transfected HeLa control cells (100%). Data are mean + S.E.M., $n = 4$. $^{***}p \leq 0.001$. (C) 5-LO-inhibitory activity of celecoxib at the concentrations indicated in 5-LO-transfected (5-LO/psg5) and 5-LO/FLAP-cotransfected (5-LO/FLAP) HeLa cells in presence of 20 μM AA. 5-LO activity was determined as described in Section 2 and expressed as percentage compared to vehicle control (100%; 25 ng/ml 5-HETE for 5-LO/FLAP cells and 19 ng/ml 5-HETE for 5-LO/psg5 cells). Data are mean + S.E.M., $n = 4$.

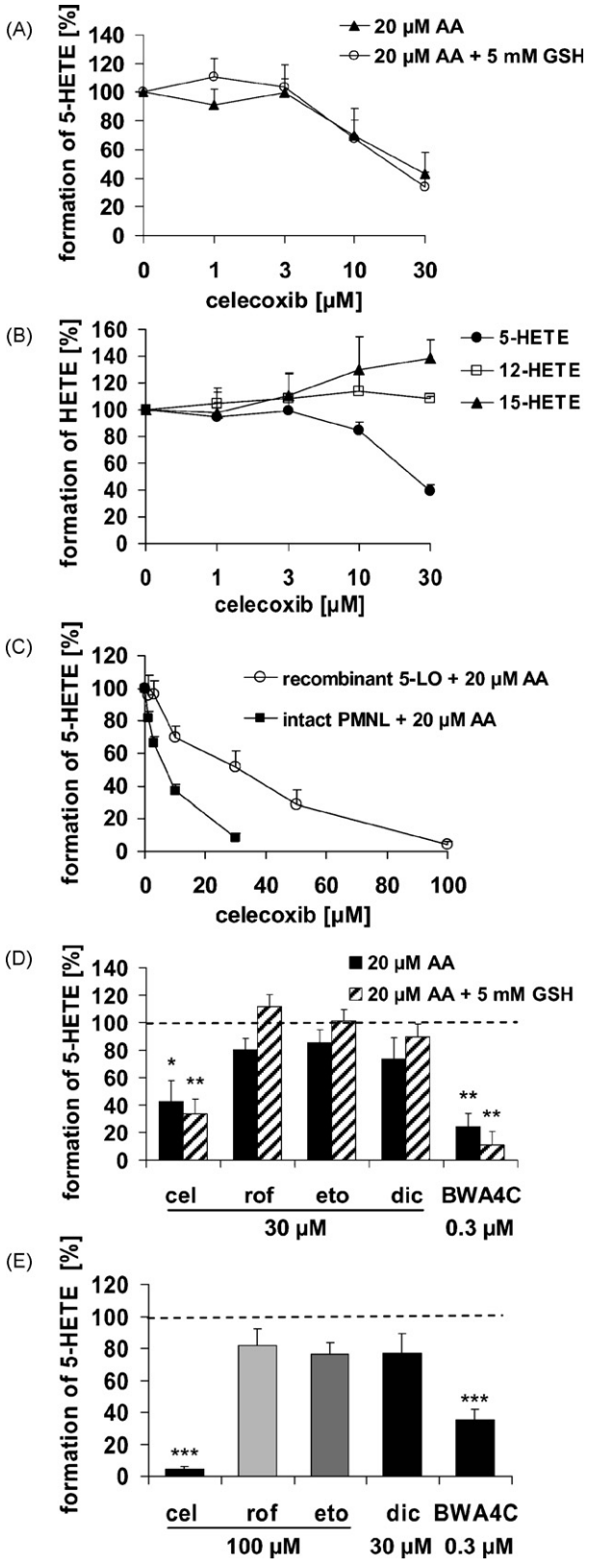

Fig. 4 – Effect of celecoxib, etoricoxib, rofecoxib, diclofenac and BWA4C on 5-LO activity in cell-free assays. 5-LO activity was determined as described in Section 2 and is expressed as percentage versus vehicle control (100%). (A) Inhibition of 5-LO product formation in broken PMNL cell homogenates by celecoxib in presence or absence of 5 mM GSH. Results are given as mean + S.E.M., $n = 4$. (B) Determination of 5-, 12- and 15-LO product formation in broken PMNL cell homogenates in the presence of increasing concentrations of celecoxib, 20 μM AA and 1 mM GSH. Results are given as mean + S.E.M., $n = 3$. (C) Inhibition of partially purified human recombinant 5-LO by celecoxib compared to intact PMNL. Results are given as mean + S.E.M., $n \leq 4$. (D) Comparison of the 5-LO-inhibitory activity of celecoxib, rofecoxib, etoricoxib, diclofenac and BWA4C in PMNL homogenates in the absence (black bars) or presence (striped bars) of 5 mM GSH versus vehicle control (100%). Results are given as mean + S.E.M., $n = 4$. (E) Effect of celecoxib, rofecoxib, etoricoxib, diclofenac and BWA4C on recombinant 5-LO activity compared to vehicle control (100%). Results are given as mean + S.E.M., $p \geq 3$. $^{***}p \leq 0.001$; $^{**}p \leq 0.01$; $^{*}p \leq 0.05$. cel, celecoxib; eto, etoricoxib; rof, rofecoxib; dic, diclofenac.

3.5. *Celecoxib dose-dependently inhibits 5-LO product formation in rats*

In order to investigate whether or not inhibition of 5-LO may occur at pharmacologically achievable celecoxib concentrations *in vivo*, we analyzed the efficacy of celecoxib in rats using an *ex vivo* whole blood assay in which zileuton and etoricoxib were used as reference drugs. After collection of the pre-dose blood, the drugs were injected i.p. After 15 min, venous blood was taken and 5-LO products were analyzed. Celecoxib led to a significant and dose-dependent reduction of both LTB_4 and 5-HETE formation with ED_{50} values of 35.2 and 59.3 mg kg^{-1}, respectively (Fig. 5A and B). It is noteworthy that the ED_{50} value of i.v. administered celecoxib to counteract carrageenan-induced edema formation (anti-inflammatory effect) in rats was reported to be 7.5 mg kg^{-1} [31], the ED_{50} of orally administered celecoxib for the formalin assay (anti-nociceptive effect) was 67.1 mg kg^{-1} [32]. In contrast, there was no significant reduction of 12-HETE and 15-HETE formation by celecoxib (data not shown) and neither low (2 mg kg^{-1}) nor high doses (30 mg kg^{-1}) of etoricoxib affected 5-LO product formation over the vehicle control (DMSO, dimethylsulfoxide) (Fig. 5A and B). The respective plasma concentrations of the drugs were analyzed using LC–MS/MS as previously described [16] (Fig. 5C). Non-linear regression of the inhibition of 5-LO product formation versus corresponding celecoxib plasma concentrations in rats yielded IC_{50} values of ~25 μM for LTB_4 and ~31.5 μM for 5-HETE indicating a similar 5-LO-inhibitory potency of celecoxib in the *in vitro* human whole blood assay (Fig. 1) and *ex vivo* in rats.

4. Discussion

Here we demonstrate for the first time that the COX-2-selective drug celecoxib directly inhibits 5-LO both *in vitro* and *in vivo*. Celecoxib inhibited 5-LO product synthesis in two different cell based contexts, that is in A23187-activated human PMNL and in transfected HeLa cells. Celecoxib efficiently reduced the blood levels of 5-LO products *ex vivo*

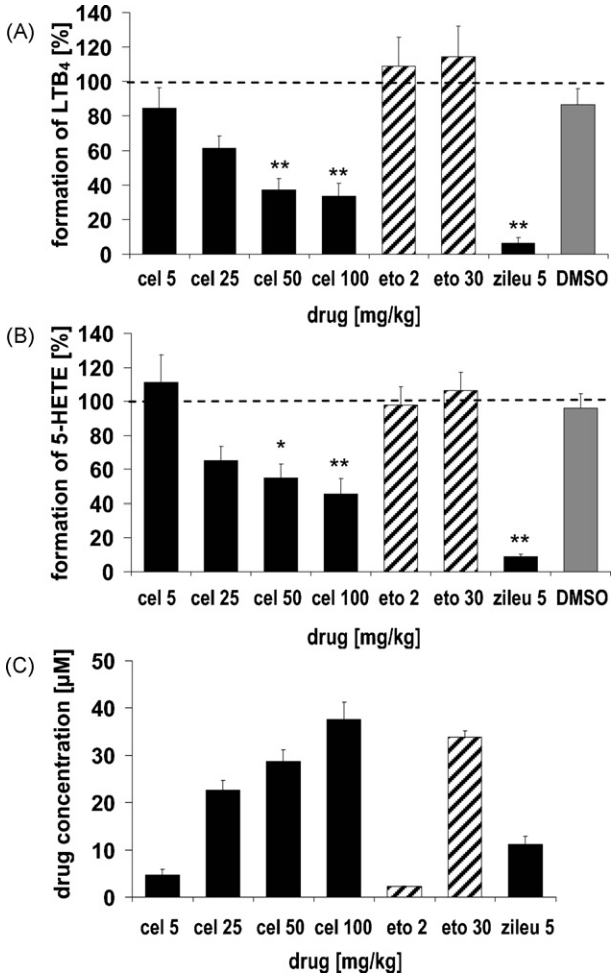

Fig. 5 – Effect of celecoxib, etoricoxib and zileuton administered by i.p. injection on 5-LO product formation in rats. 5-LO product formation in the pre-dose blood was set to 100% and compared to the respective formation in after-dose blood. *Ex vivo* whole blood assay procedure was performed as described in Section 2. The eicosanoids in the plasma supernatant analyzed by LC–MS/MS were LTB_4 (A) and 5-HETE (B). (C) Plasma drug concentrations determined by LC–MS/MS analysis achieved after i.p. injection into rats. Results are given as mean + S.E.M. of 5 (cel 100 mg/kg, cel 50 mg/kg, eto 30 mg/kg, DMSO; n = 8–10 different rats), 4 (cel 25 mg/kg, cel 5 mg/kg; n = 6–8 different rats) and 2 (zileu 5 mg/kg, eto 2 mg/kg; n = 6–7 different rats) independent experiments. Significant differences in 5-LO product formation of treated animals versus animals that received vehicle (DMSO) are indicated with an asterisk; $^{**}p \leq 0.01$; $^{*}p \leq 0.05$. cel, celecoxib; eto, etoricoxib; rof, rofecoxib; dic, diclofenac; zileu, zileuton.

after i.p. administration of the drug at standard doses to rats, suggesting a pharmacological relevance of 5-LO inhibition, at least in animal studies. A direct interaction between 5-LO and celecoxib was visualized in cell-free assays, where celecoxib blocked the activity of isolated human recombinant 5-LO or 5-LO in human PMNL homogenates. Nevertheless, celecoxib may operate at yet unrecognized cellular mechanisms which enhances the potency to suppress 5-LO product synthesis in intact cells versus cell-free assays. Regarding animal models our data strongly suggest that achievable plasma concentrations in rats after reasonable doses of celecoxib affect 5-LO activity in leukocytes and may thus contribute to the pharmacodynamic profile of this drug.

5-LO contains a non-heme iron in the active site that cycles between the ferrous and the ferric state [33]. Most pharmacological 5-LO inhibitors target this iron by preventing oxidation of the ferrous state and/or by chelation. Nonredox-type inhibitors compete with fatty acid binding site(s) [33]. Based on the structure of celecoxib, an iron-chelating or a redox-related mechanism for inhibition is considered unlikely and celecoxib also does not fit the traditional pattern of a nonredox-type 5-LO inhibitor. Possibly, 5-LO inhibition may also occur through a novel mechanism that could include interruption of 5-LO binding of stimulatory co-factors such as Ca^{2+}, ATP, lipid hydroperoxides, coactosine-like protein or phospholipids [27]. Future studies will address these alternative mechanisms in more detail. Interference of FLAP (by e.g. MK-886) is an alternative route of blocking cellular 5-LO product formation. Though the cellular environment governs inhibition of 5-LO product synthesis by celecoxib, FLAP is apparently not instrumental for celecoxib's inhibitory activity in intact cells as demonstrated by 5-LO/FLAP-cotransfection experiments. Celecoxib inhibited 5-LO product synthesis in PMNL equally well regardless whether AA was provided from endogenous sources via $cPLA_2$-alpha or exogenously supplemented in excess, suggesting that celecoxib should not reduce the supply of AA. Furthermore, the 5-LO subcellular redistribution to the nuclear membrane in response to A23187 was not affected by celecoxib. Finally, interference with MAPK and 5-LO phosphorylations is unlikely, since A23187 was used to activate PMNL that circumvents receptor-coupled signalling and thus causes MAPK/phosphorylation-independent 5-LO activation in PMNL [17].

A number of non-COX-2 targets of celecoxib were identified, responsible for its pharmacological profile including a distinctive chemo-preventive and tumor-regressive efficacy at rather high celecoxib doses (~10–150 mg/kg/d for several weeks in animal models) as well as a favourable gastrointestinal tolerability compared to classical NSAIDs. Celecoxib's pharmacological actions have been attributed to numerous COX-independent mechanisms: inhibition of 3-phosphoinositide-dependent kinase-1 (PDK-1, IC_{50} = 48 μM, intact cells) [10], inhibition of endoplasmatic reticulum $Ca^{-(2+)}$-ATPase ($IC_{50} \approx 35$ μM) [11] in human prostate cancer cells, degradation of the oncogenic survival factor beta-catenin in human colon carcinoma cells (at 60–100 μM) [12], and inhibition of adenylyl-cyclases (IC_{50} = 375 μM) [13]. However, the relevance of these findings is greatly debated because of a strong discrepancy between the plasma concentrations of celecoxib in humans after intake of 400 mg bid (recommended dose for FAP patients, $c_{max} \approx 7.4$ μM) [34] and those concentrations ($IC_{50} \geq 35$ μM) required to affect non-COX-2 targets *in vitro*. Inhibition of cellular 5-LO product synthesis occurs at lower celecoxib concentrations ($IC_{50} \approx 8$ μM), and regarding the efficacy in whole blood (Fig. 1), a reduction of LTB_4 synthesis by 15–20% can be expected at celecoxib steady state plasma concentrations under dosage regimes of 200 or 400 mg

bid. However, for distinctive reduction of 5-LO product levels, celecoxib concentrations are necessary that almost 4-fold exceed the plasma drug concentrations in patients. Yet, the relevance of this discrepancy is arguable, because patient therapy regimes typically require weeks or months of drug treatment whereas the incubation time of celecoxib in our whole blood assays was for only 30 min. Thus, the pharmacological relevance of 5-LO inhibition by celecoxib at tolerated doses in humans requires further elucidation.

During the preparation of this manuscript, a paper was published demonstrating the suppression of 5-LO products synthesis by celecoxib in A23187-stimulated human PMNL and in human whole blood, which is in line with our findings. However, no mechanistic analysis of the interference of celecoxib with 5-LO or with any other element within 5-LO product biosynthesis was performed by these authors [35]. Also, Chen et al. demonstrated that high doses of celecoxib in chow (1000 ppm) reduced the content of LTB_4 by approx. 50% in oesophageal adenocarcinoma xenografts of rats, while lower doses were almost ineffective [36]. A 50% reduction of LTB_4 and 5-HETE formation in A23187-stimulated human whole blood by 100 μM celecoxib has also been described, albeit under different experimental conditions than the present study [37]. On the other hand, Gyllfors et al. showed that 400 mg celecoxib (as 200 mg bid) administered to subjects with aspirin-intolerant asthma, caused no changes in urinary LTE_4 levels [38]. Furthermore, Mao et al. showed that administration of celecoxib 400 mg twice daily to active smokers increased the production of LTB_4 in broncho alveolar lavage fluids by around 36% [39]. Thus, modulation of 5-LO product synthesis by celecoxib may depend on various factors including smoking, the state of health of the subject, the drug dosage and the duration of the treatment regime.

A crucial role of 5-LO in tumor development has been well established. Distinct inhibition of tumor growth through 5-LO inhibition was reported for human prostate, oesophageal, pancreatic, lung and colorectal cancers [40]. Interestingly, celecoxib-mediated antiproliferative effects are mechanistically similar to those observed during 5-LO inhibitor-triggered cell death. For instance, both 5-LO inhibitors and celecoxib attenuate the growth of human carcinoma cells through the intrinsic pathway of apoptosis and through inducing the cell cycle inhibitor $p21^{kip1}$ [8,41,42]. Moreover, inhibition of 5-LO down regulates the LTD_4-mediated beta-catenin-signalling [43] and this pathway is suppressed also by celecoxib [12]. Finally, LTB_4 causes Akt kinase activation by PI-3-kinase/PDK-1-mediated phosphorylation [44], suggesting that inhibition of LTB_4 formation by celecoxib may contribute to the down-regulation of cellular Akt activity described for this drug a few years ago [10].

Accumulating evidence suggests 5-LO as a key player within the arterial wall during human atherogenesis and 5-LO products were identified as key regulators of smooth muscle cell proliferation and of migration, which causes plaque instability [45,46]. Thus, anti-LT drugs were considered to be a novel therapeutic option for the treatment of atherosclerosis [47]. A higher incidence of adverse cardiovascular side effects was observed after long term treatment with selective as well as unselective COX inhibitors and there is a wide spread agreement that the cardiovascular toxicity reflects a class effect of all COX-2-selective drugs [48]. Detrimental cardiovascular side effects were clearly demonstrated for rofecoxib and other COX-2 inhibitors, whereas the data on celecoxib are still heterogeneous [49–53]. It is therefore speculative, at present, to establish for celecoxib a clear connection between the 5-LO-inhibitory activity and a possible favourable cardiovascular profile.

In conclusion, celecoxib inhibits 5-LO in cell-free assays, in cellular systems, in human whole blood as well as *in vivo* in rats and this inhibition is unique for celecoxib amongst the other coxibs tested. Furthermore, our mechanistic studies revealed a direct inhibitory interaction between celecoxib and the 5-LO enzyme. Interestingly, the COX-2-selective inhibitor lumiracoxib was recently found to display antagonism of human thromboxane receptors, supporting the present idea that COX inhibitors concurrently may affect other enzymes that bind or convert AA-derived metabolites [54]. Further studies addressing the effects of celecoxib on the LT biosynthesis in patients at clinical standard dosage regimes for prolonged periods will be a step forward in order to better understand the pharmacological profile of this drug.

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