ABSTRACT

Lipoxygenases (LOs) are iron-containing enzymes that catalyze the conversion of arachidonic acid into hydroperoxyeicosatetraenoic acids (H-PETEs) and other bioactive lipid mediators. In mammals, 5-LO, 15-LO, and 12-LO enzymes seem to have distinct roles in pathophysiological contexts, which have emphasized the need for selective inhibitors. Cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC) has been proposed as potent and selective inhibitor of platelet-type 12-LO (p12-LO). Here, we re-evaluated the selectivity profile of CDC on LOs, and we show that CDC is a potent and direct inhibitor of 5-LO. CDC reduced 5-LO activity in cell-free assays (purified human leukocytes or leukocyte homogenates), with IC_{50} values in the low nanomolar range (9–25 nM) and a selectivity index of approximately 35 and 15 over p12-LO and 15-LO1, respectively. Likewise, CDC inhibited 5-LO product formation in intact human polymorphonuclear leukocytes and monocytes (IC_{50} = 0.45–0.8 μM). A lower potency was observed for 15-LO1, whereas p12-LO activity in platelets was hardly affected. In human whole blood, CDC efficiently reduced the formation of 5-LO products, and similar effects were observed for 12(5S)-P(II)ETE and 15(5S)-P(II)ETE. Finally, CDC (3.5 and 7 mg/kg i.p.) was effective in vivo in the platelet-activating factor-induced shock in mice and reduced formation of the 5-LO product leukotriene B_{2} in the rat carrageenan-induced pleurisy after a single oral dose of 10 mg/kg. Together, our data demonstrate that CDC is a potent inhibitor of 5-LO with efficacy in vivo and encourage further development of CDC as the lead compound.

Introduction

Lipoxygenases (LOs) are structurally related dioxygenases that catalyze the incorporation of molecular oxygen into polyunsaturated fatty acids containing a (Z,Z)-1,4-pentadiene system (Brash, 1999). In mammals, arachidonic acid (AA) is predominantly used as substrate, and the formed hydro(pero)xyeicosatetraenoic acids [H(P)ETEs] can then be reduced to the corresponding hydroxy compounds or be further metabolized [e.g., to leukotrienes (LTs)]. Therefore, LOs represent important sources of lipid mediators, with critical roles in both physiological and pathological contexts (Yoshimoto and Henderson, 2007). The LO reactions are region-specific and, according to the positional specificity on AA, 5-LO, 12-LO, and 15-LO have been recognized in humans.

5-LO is highly expressed in polymorphonuclear leukocytes (PMNL) and monocytes (Rådmark et al., 2007). Its products, 5(S)-H(P)ETE and LTs, are mediators of immune and inflammatory responses, and inhibition of 5-LO is a strategy to intervene with allergic and inflammatory disorders (e.g., asthma) (Pergola and Werz, 2010) and also possibly with atherosclerosis (Bäck, 2009). Human blood PMNL and monocytes may also contain 15-LO1 (Nadel et al., 1991; Kim et al., 1995), which is closely related to the murine leukocyte-type 12-LO (also known as 12/15-LO). These enzymes can metabolize AA to 12(S)-H(P)ETE and to 15(S)-H(P)ETE and may directly modify the organization of membranes, and their products can interact with several inflammation-related mediators. 

Cinnamyl-3,4-Dihydroxy-α-Cyanocinnamate Is a Potent Inhibitor of 5-Lipoxygenase

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pathways, with roles in atherogenesis, chemotaxis, and cancer growth (Kuhn and O'Donnell, 2006). The platelet-type 12-LO (p12-LO) instead metabolizes AA predominantly to 12(S)-HETE (Yoshimoto and Takahashi, 2002). Despite possible roles of p12-LO in platelet aggregation, atherosclerosis, angiogenesis, cancer metastasis, and cell migration, the exact contribution of p12-LO to human physiology and pathophysiology still remains elusive.

For this reason, research has extensively focused on the identification of selective p12-LO inhibitors as tools to identify specific actions of p12-LO and lead compounds in drug development. However, only few p12-LO inhibitors with a selectivity index >10 over 5-LO and 15-LO have been identified. Among the different strategies used, structural modification of known 5-LO inhibitors to gain selectivity on p12-LO has been reported to be successful (Cho et al., 1991). Thus, a series of 3,4-dihydrorxy-α-cyanocinnamoyl esters derived from the redox-type 5-LO inhibitor caffeic acid have been claimed as potent and selective p12-LO inhibitors. In particular, the synthetic compound, cinnamyl-3,4-dihydrorxy-α-cyanocinnamate (CDC) (Fig. 1), was reported to inhibit p12-LO with an IC50 of 63 nM and to be selective approximately 30 and 53 times over 5- and 12/15-LO, respectively. Therefore, this compound has been used as a pharmacological tool to identify 12-LO-mediated action and is currently considered as a rather selective 12-LO inhibitor (Stern et al., 1999; Wen et al., 1996; Kuwata et al., 1998; Kalvegren et al., 2007; de Carvalho et al., 2008; Guo et al., 2011).

However, the selectivity profile of CDC was analyzed so far only in cell-free assays using crude homogenates of rat platelets and rat PMNL as systems to test the effects on p12-LO and on 5- and 12/15-LO, respectively. Furthermore, the assay conditions for the different enzymes (e.g., AA concentrations, presence of glutathione) were inconsistent and did not consider pharmacologically relevant factors that may be relevant in intact cells and/or in vivo. In this study, we provide a detailed evaluation of the effectiveness of CDC to inhibit human 5-LO, 15-LO1, and p12-LO in selected well-established and defined test systems, taking into account essential parameters that affect the efficiency in vivo.

Materials and Methods

Materials. CDC was from Enzo Life Sciences (Lorrach, Germany). Zileuton [N-(1-benzol[b]thien-2-yleyl]-N-hydroxyurea] was from Sequoia Research Products (Oxford, UK). Platelet-activating factor (PAF, C-16) was from Cayman Chemical (Ann Arbor, MI). α-Carrageenan type IV (isolated from Gigartina aciculaire) was from Sigma-Aldrich (Milan, Italy). All other chemicals were purchased from Sigma-Aldrich, unless stated otherwise. HPLC solvents were from Merck (Darmstadt, Germany).

Expression and Purification of 5-LO, p12-LO, and 15-LO1. 5-LO was expressed in Escherichia coli Bl21 (DE3) cells transformed with pT3-5LO, and purification of 5-LO was performed as described previously (Fischer et al., 2003). In brief, E. coli were harvested by centrifugation (7709g for 15 min) and lysed in 50 mM triethanolamine/HCl, pH 8.0, 5 mM EDTA, 60 μg/ml soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 1 mg/ml lysozyme; homogenized by sonication (3 × 15 s); and centrifuged at 10,000g for 15 min followed by centrifugation at 40,000g for 70 min at 4°C. The supernatant was then applied to an ATP-agarose column (Sigma-Aldrich), and the column was eluted as described previously (Brungs et al., 1995). Partially purified 5-LO was immediately used for activity assays.

p12-LO was expressed in E. coli Bl21 (DE3) cells transformed with pT3-p12-LO (provided by Dr. Colin Funk, Kingston, Canada), and 15-LO1 protein was expressed in E. coli Bl21 (DE3) cells transformed with pQE-9-his-15-LO1 (Dr. Hartmut Kuhn, Berlin, Germany). Purification of his-p12-LO and his-15-LO1 was performed using nickel-affinity chromatography. In brief, E. coli were harvested and lysed as described for 5-LO. The resulting supernatant (S400) was then mixed with nickel-nitriotriacetic-agarose beads (QIAGEN GmbH, Hilden, Germany) in a ratio of 1:200 (for p12-LO) or 1:40 (for 15-LO1) and incubated overnight at 4°C under rotation. Beads were then washed three times for 5 min on ice with 50 mM NaH2PO4 and 300 mM NaCl containing 50 mM imidazole (for p12-LO) or 20 mM imidazole (for 15-LO1) followed by wash three times for 5 min with elution buffer (50 mM NaH2PO4, 300 mM NaCl, and 250 mM imidazole). Centrifugation at 12000g was performed after each step. Eluted fractions were pooled and used immediately for p12-LO and 15-LO1 activity assays, respectively.

Diphenylpicrylhydrazyl Assay. The antioxidant activity of CDC was assessed by the method of Blois (1958), with slight modifications. In brief, 100 μl of 1, 2.5, 5, 10, 25, 50, or 100 μM CDC in ethanol (corresponding to 0.1, 0.25, 0.5, 1, 2.5, 5, or 10 nmol) were added to 100 μl of a solution of the stable free radical diphenylpicrylhydrazyl (DPPH) in ethanol (100 μM, corresponding to 10 nmol) buffered with acetate to pH 5.5 in a 96-well plate. The absorbance was read at 520 nm after a 30-min incubation under gentle shaking in the dark. Ascorbic acid and l-cysteine were used as reference compounds and reduced the DPPH radical with a 1:2 (ascorbic acid/DPPH) and 1:1 (l-Cys/DPPH) apparent stoichiometry (data not shown). All of the analyses were performed in triplicates.

Cells. Human PMNL, platelets, and monocytes were freshly isolated from leukocyte concentrates obtained at the Blood Center, University Hospital (Tuebingen, Germany). In brief, venous blood was collected from fasted (12 h) adult female healthy volunteers with consent. The subjects had no apparent inflammatory conditions and had not taken anti-inflammatory drugs or anticoagulants for at least 10 days before blood collection. Venous blood was subjected to centrifugation at 4000g, 20 min, 20°C for preparation of leukocyte concentrates. PMNL, platelet-rich plasma, and peripheral blood mononuclear cells (PBMC) were separated by dextran sedimentation and centrifugation on Nycosrep cushions (PAA Laboratories, Linz, Austria). For isolation of PMNL, pelleted cells were collected, and hypotonic lysis of erythrocytes was performed as described previously (Werz et al., 2002). For isolation of platelets, platelet-rich plasma was obtained from the supernatants, mixed with phosphate-buffered saline (PBS), pH 5.9 (3:2 v/v), and centrifuged (2100g, 15 min, room temperature). The pelleted platelets were resuspended in PBS, pH 5.9/0.9% NaCl (1:1, v/v), centrifuged again, and resuspended in PBS pH 7.4. For isolation of human monocytes, the PBMC fractions were collected and washed three times with ice-cold PBS, and then monocytes were separated by adherence for 1.5 h at 37°C to culture flasks (Greiner, Nuertingen, Germany; cell density was 2 × 107 cells/ml RPMI 1640 medium containing 2 mM L-glutamine and 50 μg/ml penicillin/streptomycin), which finally gave a purity of approximately 80%, defined by forward- and side-light scatter properties and detection of the CD14 surface molecule by flow cytometry (BD FACSCalibur; BD Biosciences, Franklin Lakes, NJ).

Determination of LO Product Formation. For assays of intact cells, 109 freshly isolated PMNL or monocytes and 109 freshly isolated platelets were finally resuspended in 1 ml of PBS plus 1 mg/ml

![Fig. 1. Chemical structure of CDC.](image-url)
CDC Inhibits 5-Lipoxygenase

were allowed to recover. Animals were killed after 2 h by inhalation of CO₂. The skin incision was closed with a suture, and the animals submitted to a skin incision at the level of the left sixth intercostal space. The chest was carefully opened, and the pleural cavity was used for assays in whole blood, aliquots of freshly withdrawn blood (2 ml) were preincubated with CDC or vehicle (0.1% DMSO) for 10 min at 37°C, and formation of LO products was started by the addition of calcimycin (AZ2187) (30 µM) in the presence or absence of 100 µM AA (Pergola et al., 2008). Samples were incubated for 10 min at 37°C, and the reaction was stopped on ice. The samples were centrifuged (600g, 10 min, 4°C), aliquots of the resulting plasma (500 µl) were then mixed with 2 ml of methanol, and 200 ng of prostaglandin B₁ were added as internal standard. The samples were placed at −20°C for 2 h and centrifuged again (600g, 15 min, 4°C). The supernatants were collected and diluted with 2.5 ml of PBS and 75 µl of 1 N HCl.

Formed 5-LO metabolites, 12(S)-H(2)ETE and 15(S)-H(2)ETE, were extracted and analyzed by HPLC as described previously (Wenz et al., 2002). 5-LO products include LTD₄ and its all-trans-isomers and 5(S)-H(2)ETE. The cysteinyl-LTs were not included in the analyses, since previous studies showed that the amount of cysteinyl-LTs formed in A23187 ionophore-stimulated blood leukocytes and whole blood is negligible (approximately 1–2% of the total amount of 5-LO products produced; Pergola et al., 2008).

For determination of LO product formation in homogenates, 1 mM EDTA was added to cells resuspended in PBS. Samples were cooled on ice (5 min) and sonicated (3 × 10 s) at 4°C. For 5-LO, 1 mM ATP was added. Samples of either partially purified enzymes (1 ml) or cell homogenates (corresponding to 2 × 10⁶ PMNL or monocytes and 10⁴ platelets in 1 ml) were incubated 10 min at 4°C with vehicle (0.1% DMSO, control) or CDC and prewarmed for 30 s at 37°C and 2 mM CaCl₂, and the indicated concentrations of AA were added. The reaction was stopped after 10 min at 37°C by the addition of 1 ml of ice-cold methanol, and 200 ng of prostaglandin B₁ were added. Formed metabolites were extracted and analyzed by HPLC as described previously (Wenz et al., 2002).

Animals. Female Wistar Han rats (220–230 g; Harlan, Milan, Italy) and female CD-1 mice (25–30 g; Harlan) were housed in a controlled environment and provided with standard rodent chow and water. All animals were allowed to acclimate for 4 days before experiments and were subjected to 12–18 h/day light/dark schedule. Experiments were conducted during the light phase. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (Ministerial Decree 116192) as well as with the European Economic Community regulations (Official Journal of E.C. L 358/1 12/18/1986).

Mouse PAF-Induced Shock. PAF C-16 was dissolved in chloroform and stored at −20°C. The PAF working solution was freshly prepared directly before use. To this aim, chloroform was evaporated under N₂, and PAF was dissolved in 0.9% saline solution containing 0.25% bovine serum albumin. Mice were challenged with 200 µg/kg PAF in a volume of 200 µl via a tail vein injection 30 min after an intraperitoneal injection of either vehicle (0.9% saline solution containing 4 or 2% DMSO as control for CDC and zileuton, respectively), CDC or zileuton, at the indicated doses. Death (determined by cessation of breathing) was then recorded over a period of 2 h. All animals surviving the 2-h test session were euthanized by CO₂ inhalation.

Carrageenan-Induced Pleurisy in Rats. CDC (10 mg/kg) or vehicle (0.5 ml of 0.5% carboxymethylcellulose and 10% Tween 20) were given orally 1 h before carrageenan. Rats were anesthetized with 4% enflurane mixed with 0.5 l/min O₂, 0.5 l/min N₂0 and submitted to a skin incision at the level of the left sixth intercostal space. The underlying muscle was dissected, and saline (0.2 ml) or 1% (w/v) λ-carrageenan type IV (0.2 ml) was injected into the pleural cavity. The skin incision was closed with a suture, and the animals were allowed to recover. Animals were killed after 2 h by inhalation of CO₂. The chest was carefully opened, and the pleural cavity was rinsed with 2 ml of saline solution containing heparin (5 U/ml). The exudate and washing solution were removed by aspiration, and any exudate that was contaminated with blood was discarded. The amounts of LTBP₁, in the supernatant of centrifuged exudate (800g for 10 min) were assayed by enzyme immunoassay (Cayman Chemical, Ann Arbor, MI) according to manufacturer’s protocol. Results are expressed as the total amount of LTBP₁ measured in the pleural exudate of one rat (nanograms per rat).

Statistics. Results are expressed as mean ± S.E.M. of the mean of n observations, where n represents the number of experiments performed on different days in duplicates or the number of animals as indicated. The IC₅₀ values were determined by linear interpolation and validated with GraphPad InStat program (GraphPad Software, Inc., San Diego, CA). Data fit was obtained using the sigmoidal dose-response equation (variable slope) (Prism software; GraphPad Software, Inc.). Statistical evaluation of the data was performed by one-way analysis of variance (ANOVA) for independent or correlated samples followed by Tukey honest significant difference post hoc tests. Where appropriate, Student’s t test for paired observations was applied. Contingency tables were analyzed with one-sided Fisher’s exact test. A p value < 0.05 was considered significant.

Results

Inhibition of Partially Purified LOs by CDC. We attempted to evaluate the molecular pharmacological profile of CDC as an inhibitor of human LOs. To this aim, we first analyzed the effect of CDC on partially purified human 5-LO, p12-LO, and 15-LO1 under standardized assay conditions (20 µM AA as substrate and 1 mM Ca²⁺ as supplement). CDC exhibited an extremely potent inhibitory activity on human 5-LO. We determined the IC₅₀ at 15 ± 5 nM (Fig. 2A), and total suppression was achieved already at 100 nM CDC. It is noteworthy that CDC was significantly less effective as a p12-LO inhibitor, and the IC₅₀ value and the concentration needed to achieve total suppression of p12-LO (500 nM and 3 µM, respectively) were approximately 30 times higher than those for 5-LO (Fig. 2A). It is noteworthy that CDC also inhibited 15-LO1 (IC₅₀ = 300 nM) with a similar potency as the p12-LO (Fig. 2A). Variations of the enzymatic activities of the LOs from batch to batch did not affect the inhibitory potencies of CDC (data not shown).

Enzymatic oxidation of AA by LOs involves the generation of free radical intermediates, and redox-active compounds can scavenge radicals and/or reduce the active site iron and thus uncouple the catalytic redox-cycle of LOs. CDC was originally developed from the redox-type 5-LO inhibitor caffeic acid, sharing the catechol moiety with the parental compound. To evaluate the redox potential of CDC, we analyzed its radical scavenging properties in the well recognized DPPH assay. CDC reduced the DPPH radicals with an apparent 1:4 stoichiometry (Fig. 2B). Typically, LO inhibitors act in a reversible manner; thus, we tested whether the effects of CDC are reversible. Partially purified 5-LO enzyme was preincubated with 0.1 µM CDC. After 10-fold dilution with assay buffer (0.1–0.01 µM CDC), 5-LO product formation was almost at the same level as that after treatment with 0.01 µM CDC without precedent dilution (Fig. 2C). Similar results were observed for p12-LO and 15-LO1 (CDC concentrations of 1 and 0.1 µM). Taken together, CDC is a direct, redox-active, reversible LO inhibitor and exhibits the following order of potency: 5-LO > 15-LO1 = p12-LO.

Inhibition of LOs by CDC in Cell Homogenates. The efficiency of LO inhibitors often depends on the assay condi-
significantly higher concentrations, with an IC$_{50}$ of 2 nM; Fig. 3, A and B). However, CDC reduced 15(H)-H$_{(P)}$ETE synthesis by 15-LO1 in PMNL homogenates at 5 nM; Fig. 3, A and B). Thus, the potency of CDC in cell homogenates is similar to that observed for the isolated enzymes.

We next evaluated the effect of CDC on LOs in intact cells. After stimulation of PMNL with A23187 ionophore, 5-LO products and 12(H)-H$_{(P)}$ETE are synthesized. CDC concentration-dependently inhibited 5-LO product formation, with an IC$_{50}$ of 0.5 ± 0.1 μM (Fig. 4A, left), and thus resulted to be more potent than the 5-LO inhibitor zileuton under the same assay condition (IC$_{50}$ = 1.7 ± 0.7 μM; Fig. 4A, right). The formation of the 5-LO metabolites LTB$_4$ and 12-H$_{(P)}$ETE was inhibited with a similar potency by CDC (data not shown). A similar effect was observed for inhibition of 12(S)-H$_{(P)}$ETE (IC$_{50}$ = 0.8 ± 0.2 μM). In the presence of exogenous AA, CDC still inhibited 5-LO (IC$_{50}$ = 0.8 ± 0.1 μM) but was significantly less effective in reducing 12(S)-H$_{(P)}$ETE formation (IC$_{50}$, approximately 2 μM) and only hardly reduced 15(S)-H$_{(P)}$ETE production (Fig. 4B). Analogous to PMNL, CDC efficiently reduced 5-LO product formation in A23187-stimulated monocytes in the presence or absence of exogenous AA (IC$_{50}$ = 0.45 ± 0.1 μM, both; Fig. 5, A and B). A similar effect was observed in A23187-stimulated cells on 12(S)-H$_{(P)}$ETE formation (IC$_{50}$ = 1.1 ± 0.2 μM; Fig. 5A). It is noteworthy that the addition of exogenous AA resulted in a significant improvement of the potency on 12(S)-H$_{(P)}$ETE formation by the leukocyte-type 12-LO (IC$_{50}$ = 0.1 ± 0.05 μM), whereas the reduction of 15(S)-H$_{(P)}$ETE was observed only at higher concentrations (IC$_{50}$ = 6 ± 1 μM) (Fig. 5B). In A23187-stimulated platelets, CDC only moderately inhibited p12-LO (IC$_{50}$ > 10 μM), whereas a
paradoxical increase of 12(S)-H(2)ETE formation was observed when cells were stimulated by exogenous AA (Fig. 5C). Finally, 12(S)-H(2)ETE formation also was evaluated after activation of platelets with more pathophysiological relevant stimuli, i.e., thrombin and collagen. Under these conditions, the inhibitory potential of CDC increased (IC50 = 0.8 ± 0.1 and 1 ± 0.1 μM for thrombin and collagen, respectively; Fig. 5C).

**Inhibition of LO Product Formation by CDC in Whole Blood.** Cell-free assays and test systems based on isolated cells represent suitable models to evaluate the interference of test compounds with selected targets. On the other hand, to take into account the influence of in vivo relevant parameters on the efficiency of a LO inhibitor, whole-blood assays include critical pharmacokinetic and pharmacodynamic variables and are considered relevant test systems for translation of drug actions in vivo. CDC concentration-dependently inhibited 5-LO product formation in A23187-stimulated whole blood (Fig. 6A, left). Compared with isolated neutrophils and monocytes, the potency was slightly reduced rather similar as that for 5-LO. Note that for inhibition of all three LOs, a plateau was reached at approximately 40 to 50% of remaining LO activity.

**CDC Protects Mice against PAF-Induced Shock and Reduces LTB4 in Carrageenan-Treated Rats.** To test whether 5-LO inhibition by CDC translates into significant functional effects in vivo, we used the model of the PAF-
induced lethal shock in mice. In fact, previous studies have established a critical role of 5-LO products in the acute PAF toxicity (Chen et al., 1994; Byrum et al., 1997) and represents a suitable model to test the in vivo efficacy of LT-synthesis inhibitors. Administration of 200 μg/kg PAF to vehicle-treated mice caused mortality in 10 of 10 animals (within 30 min). CDC at the dose of 3.5 mg/kg, but not at 1 mg/kg i.p., led to a survival of 40% of the animals (Table 1). The survival rate was not further improved by doubling the dose (7 mg/kg i.p.). Treatment with the 5-LO inhibitor zileuton at the dose of 20 mg/kg i.p. before PAF injection also resulted in improvement in survival of 40% (Table 1). Finally, we tested the effect of CDC on the levels of the 5-LO product LTB4 in an in vivo model of carrageenan-induced pleurisy in rats. The administration of a single oral dose of 10 mg/kg CDC to rats 1 h before carrageenan injection. Data are expressed as percentage of control, means ± S.E.M.; n = 7, each. ***, p < 0.001, Student's t test.

Fig. 6. Inhibition of LO product formation by CDC in human whole blood and in carrageenan-treated rats. LO product formation in human whole blood, after preincubation (10 min, 37°C) with CDC, zileuton, or vehicle (DMSO, 0.1%) in PGC buffer and stimulation with 30 μM A23187 (A) or 30 μM A23187 + 100 μM AA (10 min, 37°C) (B). Data are expressed as percentage of control, means ± S.E.M.; n = 3, duplicates. 5-LO products, *, p < 0.05, **, p < 0.01, ††, p < 0.001 versus 100% control; 12(S)-H(2)ETE, ††, p < 0.01 versus 100% control; 15(S)-H(2)ETE, ††, p < 0.01 versus 100% control; ANOVA + Tukey. LO products in 100% controls were (nanograms per milliliter plasma): A23187: 5-LO, 171.8 ± 68.3; 5-LO, 355.7 ± 189.9; 12(S)-H(2)ETE, 3101.8 ± 1832.6; 12(S)-H(2)ETE, 359.4 ± 289.9; 15(S)-H(2)ETE, 44.6 ± 8.4; 15(S)-H(2)ETE, 11.9 ± 3.9. C. Inhibition of 12(S)-H(2)ETE product formation in intact platelets after preincubation (10 min, 37°C) with the indicated concentrations of CDC or vehicle (DMSO) in PGC buffer and stimulation with 2.5 μM A23187, 3 μM AA, 8 μg/ml collagen, or 1 U/ml thrombin (10 min, 37°C). Data are expressed as percentage of control, means ± S.E.M.; n = 3, duplicates. Thrombin, collagen, **, p < 0.01, ††, p < 0.001 versus 100% control; A23187, ††, p < 0.01 versus 100% control; ANOVA + Tukey. 12(S)-H(2)ETE in 100% controls was (nanograms per milliliter, 10^6 cells/ml): A23187, 408.9 ± 77.4; AA, 311.6 ± 55.7; collagen, 120.6 ± 34.4; thrombin, 241.1 ± 31.4.
TABLE 1

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<th>CDC protects mice against PAF-induced shock</th>
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<td>PAF was injected into the tail vein of female mice at a dose of 200 μg/kg. CDC (1, 3.5, and 7 mg/kg), zileuton (10 mg/kg), or vehicle control (0.9% saline solution containing 4% DMSO or 2% DMSO, as control for CDC and zileuton, respectively) was administered intraperitoneally 30 min before PAF. The number of surviving animals and the total number of animals tested for each group are given.</td>
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* p = 0.0433, one-sided Fisher’s exact test.

Discussion

In this study, we analyzed the inhibitory actions and the selectivity profile of CDC on human 5-LO, 15-LO1, and p12-LO. On the basis of previous results by others obtained in rat cell homogenates, CDC so far has been considered as a potent and selective p12-LO inhibitor and as such used as a pharmacological tool to establish roles of p12-LO in biological processes. Our data confirm that CDC is a direct inhibitor of human LOs but demonstrate that 5-LO and not p12-LO is the preferred target of CDC in both cell-free and cell-based assays. It is noteworthy that inhibition of 5-LO by CDC was also evident in whole blood and that CDC was effective in vivo in the PAF-induced shock in mice and reduced LTB4 levels in carrageenan-treated rats. On the basis of its ability to inhibit 5-LO in various biological assays and the efficacy in animal models, we suggest further analysis to exploit the pharmacological potential of CDC. On the other hand, the potent inhibition of 5-LO by CDC together with its redox features suggest caution in interpreting results of pharmacological studies where CDC is used as a tool to tie roles of p12-LO in biological processes.

LOs are AA-metabolizing enzymes implicated in several diseases, including inflammation and cancers (Yoshimoto and Takahashi, 2002; Kuhn and O’Donnell, 2006; Peters-Golden and Henderson, 2007). To explore the respective contributions of 5-LO, 15-LO, and p12-LO in physiological and pathophysiological states and to pursue rational drug design selective inhibitors would be valuable but many developed compounds act on more than one LO isoform. LOs are in fact all iron-containing redox-active enzymes and share AA as substrate, which may bind to a common, more or less conserved AA-binding pocket at the active site. With regard to inhibition of p12-LO, the flavonoid baicalein, which classically has been considered a selective p12-LO inhibitor (IC50 = 0.64 μM) (Sekiya and Okuda, 1982), displays a similar inhibitory potency on 15-LO (IC50 = 0.63 μM) (Deschamps et al., 2006) and also inhibits cellular 5-LO product formation (IC50 = 7.13 μM) (Kimura et al., 1985). Likewise, gallatechin gallate, which is selective for p12-LO compared with 15-LO (IC50 = 0.14 and >100 μM, respectively), effectively also inhibits 5-LO activity (IC50 = 2 μM) (Yamamoto et al., 2005). In recent work from Suzuki et al. (2000), the tropolone hinokitiol was identified as a potent and selective p12-LO inhibitor (IC50 = 0.1 μM for p12-LO, 17 μM for 5-LO, 50 μM for leukocyte-type 12-LO, and >100 μM for 15-LO1). However, this compound undergoes a fast photochemical degradation by sunlight, which hampered its use as a lead compound. In addition, five novel 12-LO inhibitors were recently identified by high-throughput screening of diverse libraries (Deschamps et al., 2007), but only the selectivity toward 15-LOs was analyzed and only on isolated enzymes, although no data are available from cell-based assays or in vivo studies.

CDC was developed from the 5-LO inhibitor caffeic acid to gain selectivity on p12-LO. Initially, CDC was found to be potent and rather selective for p12-LO over 5-LO (5-LO/p12-LO IC50 ratio = 30) and 12/15-LO (12/15-LO/p12-LO IC50 ratio = 53). In this initial study, the compound was evaluated in homogenates of platelets and PMNL from rats in the presence of 0.05 μCi of [14C]AA and 1 mM glutathione for p12-LO and the presence of 0.2 μCi of [14C]AA for 5-LO and 12/15-LO. The formed AA metabolites were quantified by thin-layer chromatography and autoradiography. In our study, we analyzed CDC using human partially purified LO enzymes and homogenates of human cells under consistent assay conditions and monitored the metabolites via HPLC. It is noteworthy that we observed a higher inhibitory potential of CDC for 5-LO (IC50 = 9–25 nM) than initially reported for rat neutrophil homogenates (IC50 = 1.89 μM) (Cho et al., 1991), whereas similar effects were found for human 15-LO1 and rat 12/15-LO (IC50 = 2 and 3.33 μM, in human and rat neutrophil homogenates, respectively). On the contrary, a significant lower potency against human p12-LO (IC50 = 0.5–1 μM) was evident in comparison to previous work in homogenates of rat platelets (IC50 = 63 nM) (Cho et al., 1991). Thus, our data indicate a divergent selectivity profile of CDC than initially reported and define 5-LO as a preferential target. The variations observed in the potency of CDC are not readily understood, although both species-related differences (homogenates of human and rat cells) and/or the different experimental conditions (e.g., amount of AA as substrate, presence of glutathione for p12-LO incubations) might be relevant. In accordance with our findings, Bürger et al. (2000) reported an IC50 = 0.3 μM for inhibition of isolated p12-LO by CDC in the presence of 100 μM AA. In addition, Tornhamre et al. (2000) determined an IC50 of 50 μM for inhibition of p12-LO in the cytosolic fraction of human platelets in the presence of 25 μM AA and 10 μM 13-hydroperoxy-9,11-octadecadienoic acid (13-HpODE). Under these conditions, however, the oxidative capacity of 13-HpODE might have strongly impaired the potency of CDC.

LOs contain a non-heme iron, and the catalytic reaction proceeds by a radical-based mechanism where the iron cycles between the ferrous and ferric state (Rådmark et al., 2007). Agents containing a catechol moiety may reduce the site-specific uncouple the catalytic cycle of LOs (Pergola and Werz, 2010). We confirmed the radical scavenging properties of CDC and thus suggest the reducing feature as the mechanism responsible for LO inhibition. Note that the catechol structure is assumed to combine antioxidant and iron-chelating properties, resulting in highly efficient inhibition of LO enzymes, but electronic and steric features as well as the hydrophobicity of the respective catechol derivative may also determine potency and selectivity (Whitman et al., 2002; Pontiki and Hadjipavlou-Litina, 2008). CDC possesses a 3,4-dihydroxystyrene structure and has a computed log P of 3.63,
and both the styrene double bond on catechol derivatives and log P values close to 4 have been reported as favorable for inhibition of 5-LO (Naito et al., 1991). It is noteworthy that the recent elucidation of the structure of human 5-LO has revealed a distinct active site compared with other LOs (Gilbert et al., 2011), which may be relevant for the selectivity profile of LO inhibitors.

Cellular LO product formation is subject to regulation by diverse proteins (e.g., 5-LO-activating protein), signaling molecules (e.g., mitogen-activated protein kinases and Ca2+), and lipids (e.g., phosphatidylycholine, diacylglycerol), which may in turn also influence the efficiency of drugs (Pergola and Werz, 2010). CDC inhibited 5-LO in both PMNL and monocytes and showed similar efficiencies as the 5-LO inhibitor zileuton. A significant inhibition by CDC was also observed for the leukocyte-type 12-LO, whereas 12(S)-H(P)ETE formation in platelets depended on the stimulation. Thus, p12-LO activity was inhibited by CDC when thrombin and collagen (and to a lesser extent ionophore) were used as stimuli, but not in presence of exogenous AA. The addition of exogenous AA also partially modified inhibition of 12(S)-H(P)ETE by CDC in neutrophils and monocytes, suggesting a possible influence of CDC on cellular AA release. CDC also significantly reduced 5-LO product synthesis in whole blood, which implicate 5-LO as a pharmacological relevant target. In this model, CDC also inhibited 12(S)-H(P)ETE and 15(S)-H(P)ETE production, with a similar potency as that for 5-LO, indicating that blood component(s) might have a stronger influence on the inhibitory activity on 5-LO than on other LOs. Finally, CDC was effective in animal models where 5-LO products play critical roles. Although the protective effect of CDC also may ultimately be related to inhibition of other LOs than 5-LO, CDC was equipotent to the 5-LO inhibitor zileuton. It should be noted that such consistent efficiency for a 5-LO inhibitor is rare, because several compounds, which potently inhibited 5-LO in isolated cells, failed in whole blood or in vivo because of high plasma protein binding or competition with blood components (e.g., fatty acids), and zileuton currently represents the only 5-LO inhibitor that could reach the market (Werz, 2002). Furthermore, redox-active polyphenols are often intensively oxidized during absorption after oral administration, but CDC was orally active in reducing LTB4 levels in carrageenan-treated rats. These data support the use of CDC as a potential lead candidate for further developments. However, it should be pointed out that the clinical systemic use of redox type 5-LO inhibitors have often been hampered by interference with other redox systems or by production of reactive radical species and consequent side effects (e.g., hemolysis or methemoglobin formation) (McMillan and Walker, 1992). Although polyphenolics with catechol rings have been shown to cause erythrocyte hemolysis and methemoglobin formation less readily than those containing phenol rings (Galati et al., 2002), an accurate analysis of the potential toxicity of CDC should be performed to justify further developments for systemic use. On the other hand, the characteristic of CDC to combine 5-LO inhibition with redox properties might be useful for topical applications, e.g., in dermatological diseases involving LTs (Wedli and Kapp, 2001; Kaplan, 2002).

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Participated in research design: Pergola, Jazzar, Rossi, Buehring, Dehm, Sautebin, and Werz.
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Wrote or contributed to the writing of the manuscript: Pergola and Werz.

Other: Northoff contributed human blood and leukocyte concentrates.

References

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