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Hyperforin is a dual inhibitor of cyclooxygenase-1 and 5-lipoxygenase

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Abstract

The acylphloroglucinol derivative hyperforin is the major lipophilic constituent in the herb *Hypericum perforatum* (St. John's wort). The aim of the present study was to investigate if hyperforin as well as extracts of *H. perforatum* can suppresses the activities of 5-lipoxygenase (5-LO) and cyclooxygenases (COX), key enzymes in the formation of proinflammatory eicosanoids from arachidonic acid (AA). In freshly isolated human polymorphonuclear leukocytes stimulated with Ca^{2+} ionophore A23187, hyperforin inhibited 5-LO product formation with IC_{50} values of about 1–2 μM, in the absence or presence of exogenous AA (20 μM), respectively, being almost equipotent to the well-documented 5-LO inhibitor zileuton (IC_{50} = 0.5–1 μM). Experiments with purified human 5-LO demonstrate that hyperforin is a direct 5-LO inhibitor ($IC_{50} \approx 90$ nM), acting in an uncompetitive fashion. In thrombin- or ionophore-stimulated human platelets, hyperforin suppressed COX-1 product (12(*S*)-hydroxyheptadecatrienoic acid) formation with an IC_{50} of 0.3 and 3 μM, respectively, being about 3- to 18-fold more potent than aspirin. At similar concentrations, hyperforin suppressed COX-1 activity in platelets in presence of exogenous AA (20 μM) as well as in cell-free systems. Hyperforin could not interfere with COX-2 product formation and did not significantly inhibit 12- or 15-LO in platelets or leukocytes, respectively. We conclude that hyperforin acts as a dual inhibitor of 5-LO and COX-1 in intact cells as well as on the catalytic activity of the crude enzymes, suggesting therapeutic potential in inflammatory and allergic diseases connected to eicosanoids.

Keywords: 5-Lipoxygenase; Leukotriene; Cyclooxygenase; Prostaglandin; Hyperforin; *Hypericum perforatum*

1. Introduction

St. John's wort (*Hypericum perforatum*) is a traditional herbal medicine effective for the treatment of mild to moderate depressive disorders [1–4], and hyperforin is assumed as one of the main active constituents of *H. perforatum* [5–8]. Besides its antidepressive activities, hyperforin possesses antibacterial activity [9,10], inhibits proliferation of peripheral blood mononuclear cells and tumor cells, and induces apoptosis [11,12]. Moreover, *in vitro*, hyperforin is a potent ligand and activator of the pregnane X receptor that regulates expression of the cytochrome P450 3A4 monooxygenase, which is involved in the oxidative metabolism of >50% of all drugs [13].

Eicosanoids represent oxygenated derivatives of AA, formed by distinct enzymatic cascades. The prostanoids, including prostaglandins (PGs), prostacyclin and thromboxanes, derive from the conversion of AA by COX, whereas leukotrienes (LTs) are formed via the 5-LO pathway [14,15]. Both, prostanoids and LTs play pivotal roles in the initiation and maintenance of allergic diseases and inflammatory processes. LTB_4 is considered as a potent chemotactic and chemokinetic agent, whereas the cysteinyl-LTs (LTC_4, D_4, and E_4) cause smooth muscle contraction and increase vascular permeability. In view of these properties, LT synthesis inhibitors are thought to possess

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Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; 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; 12-HHT, 12(*S*)-hydroxy-5-*cis*-8,10-*trans*-heptadecatrienoic acid; LO, lipoxygenase; LPS, lipopolysaccharide; LT, leukotriene; NSAIDs, nonsteroidal antiinflammatory drugs; PBS, phosphate-buffered saline, pH 7.4; PG, prostaglandin; PGC buffer, PBS containing 1 mg/mL glucose and 1 mM $CaCl_2$; PMNLs, polymorphonuclear leukocytes; TGFβ, transforming growth factor beta.

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therapeutic potential for the treatment of asthma and inflammatory diseases [16].

Prostanoids are not only important effectors of inflammatory reactions, but also exert various physiological functions, particularly in the kidney and in the gastrointestinal systems. Thus, general inhibition of prostanoid synthesis is not devoid of side effects. Regarding prostanoid synthesis, a distinction must be drawn between the key enzymes COX-1 and -2, which show different tissue distribution and sensitivity towards inhibitors (for review on COX see Ref. [14]). Whereas the constitutively expressed COX-1 is assumed to be more responsible for the prostanoid formation under physiological conditions, the inducible COX-2 appears to be the dominant isoform in inflamed tissues producing the proinflammatory prostanoids, although recent results indicate that also COX-2 is constitutively expressed and COX-1 expression can be induced at sites of inflammation (for review, see Ref. [17]). Nevertheless, pharmacological intervention with COX enzymes has been proven beneficial in acute and menstrual pain, osteoarthritis, and rheumatoid arthritis [18].

Tremendous efforts have been spent on the development and screening of compounds for pharmacological intervention with the biosynthesis of eicosanoids. For intervention with LT synthesis, many compounds proved to be potent and selective inhibitors of 5-LO *in vitro*, but could not enter the market due to severe side effects or inefficacy in clinical trials [16]. Nonsteroidal antiinflammatory drugs (NSAIDs) that inhibit COX enzymes are the main drugs used to suppress fever and pain and to reduce the severity of inflammatory processes [19]. Considerable interest has been generated in drugs capable to inhibit equally well both COX and 5-LO pathways (dual inhibitors). Such compounds indicate broad-range antiinflammatory effectiveness devoid of gastric toxicity and renal damage [20,21]. Here we demonstrate that hyperforin acts as a dual inhibitor of COX-1 and 5-LO that potently suppresses the formation of prostanoids and LTs in intact cells as well as in cell-free systems.

2. Materials and methods

2.1. Materials

Hyperforin sodium salt was kindly provided by Dr. S.S. Chatterjee. *Hypericum perforatum* extracts were obtained from Lichtwer and Dr. Willmar Schwabe GmbH&Co. Diclofenac was a generous gift from Ciba-Geigy AG; TGFβ was purified from outdated platelets as described [22], Calcitriol was a gift from Schering AG. Monoclonal COX-2 antibody and 6-keto $PGF_{1\alpha}$ were from Cayman Cemicals. Materials used: Nycoprep, PAA Laboratories; Ca^{2+} ionophore A23187, AA, LPS, and thrombin, were from Sigma, and HPLC solvents and aspirin were from Merck.

2.2. Cells

MM6 cells were cultured as described [23]. Human platelets and polymorphonuclear leukocytes (PMNLs) were freshly isolated from leukocyte concentrates obtained at St. Markus Hospital (Frankfurt, Germany). In brief, venous blood was taken from healthy adult donors and leukocyte concentrates were prepared by centrifugation (4000 *g*, 20 min, 20°). PMNLs were immediately isolated by dextran sedimentation, centrifugation on Nycoprep cushions (PAA Laboratories), and hypotonic lysis of erythrocytes as described previously [24]. PMNLs (7.5×10^6 cells/mL; purity >96–97%) were finally resuspended in PGC buffer as indicated.

Platelets were isolated from supernatants (800 *g*, 10 min, room temperature (rt)) after centrifugation of leukocyte concentrates on Nycoprep cushions (see earlier) to obtain platelet-rich plasma. Platelet-rich plasma was then mixed with PBS, pH 5.9 (3:2, v/v), centrifuged (2000 *g*, 15 min, rt), and the pelleted platelets were resuspended in PBS, pH 5.9/0.9% NaCl (1:1, v/v), washed by centrifugation (2000 *g*, 10 min, rt), and finally resuspended in PBS, pH 7.4.

2.3. Determination of 5- and 15-LO products in PMNLs

To assay 5- and 15-LO product formation in intact cells, 7.5×10^6 freshly isolated PMNLs were finally resuspended in 1 mL PGC buffer. After preincubation with the test compounds for 15 min at 37°, the reaction was started by addition of 2.5 μM ionophore A23187 in presence or absence of exogenous AA (20 μM). After 10 min at 37°, the reaction was stopped with 1 mL of methanol and 30 μL of 1N HCl, and 200 ng PGB_1 and 500 μL of PBS were added. Formed AA metabolites were extracted and analyzed by HPLC as described [25].

When cell homogenates were assayed, the cells were finally taken up in 1 mL PBS and cooled on ice. After addition of EDTA (1 mM), sonication (3 times 5 s), addition of 1 mM ATP and the test compounds (5–10 min at 4°), the samples were preincubated for 30 s at 37° and the incubation was started by the addition of $CaCl_2$ and AA (2 mM and 20 μM final concentrations, respectively). After 10 min at 37°, the incubation was stopped with 1 mL methanol and the formed lipoxygenase products were extracted and analyzed by HPLC as described for the intact cells.

To determine product formation of purified 5-LO enzyme, 5-LO (0.1 μg in 10 μL) was added to 990 μL PBS, containing 1 mM EDTA, 25 μg/mL phosphatidylcholine, and 1 mM ATP on ice and the indicated compounds were added. After 5–10 min on ice, the samples were preincubated for 30 s at 37° and $CaCl_2$ and AA (2 mM and 20 μM, respectively) were added to start the 5-LO reaction. After 10 min the incubation was terminated, and 5-LO product formation was determined as described for the intact cells.

15-LO product formation is expressed as nanograms of 15(*S*)-hydroxy-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid (15-HETE) per 10^6 cells. 5-LO product formation is expressed as nanograms of 5-LO products per 10^6 cells which include LTB_4 and its all-*trans* isomers, 5(*S*),12(*S*)-di-hydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid, and 5(*S*)-hydro(peroxy)-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid. Cysteinyl-LTs (LTC_4, D_4, and E_4) were not detected and oxidation products of LTB_4 were not determined.

2.4. Determination of 12-LO and COX-1 product formation in platelets

To determine 12-LO and COX-1 product formation in intact cells, platelets (1×10^8, resuspended in 1 mL PGC buffer) were preincubated for 15 min with the test compounds at room temperature and the indicated stimuli were added. After 10 min at 37°, incubations were stopped by addition of 1 mL of methanol and 30 μL of 1N HCl, 200 ng of PGB1 (internal standard) and 500 μL of PBS were added. After centrifugation (800 *g*, 10 min), 12(*S*)-hydroxy-5,8-*cis*-10-*trans*-14-*cis*-eicosatetraenoic acid (12-HETE) and 12(*S*)-hydroxy-5-*cis*-8,10-*trans*-heptadecatrienoic acid (12-HHT) were extracted using C-18 solid phase extraction columns and analyzed by HPLC as described [25].

For determination in cell homogenates, platelets (1×10^8) were resuspended in 1 mL PBS (containing 1 mM EDTA) and cooled on ice for 5 min. After sonication (3×5 s), the test compounds were added (5–10 min at 4°), the samples were preincubated for 30 s at 37° and the incubation was started by the addition of Ca^{2+} and AA (2 mM and 20 μM final concentrations, respectively). After 10 min at 37°, the incubation was stopped with 1 mL of methanol and the formed 12-HETE and 12-HHT were extracted and analyzed as described for intact cells.

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

Expression of 5-LO was performed in *E. coli* JM 109 cells, transfected with pT3-5LO, as described [26]. Cells were harvested by centrifugation at 10,000 *g* for 9 min and the pellet was lysed by incubation in 50 mL 0.050 M triethanolamine/HCl pH 8.0, 5 mM EDTA, soybean trypsin inhibitor (60 μg/mL), 1 mM phenylmethylsulfonyl fluoride and lysozyme (500 μg/mL) at room temperature for 5 min followed by 25 min on ice. Lysed cells were homogenized by sonication (3×15 s) and centrifuged at 19,000 *g* for 15 min. The supernatant was collected, whereas the pellet was subjected to a second round of lysis and sonication in half the buffer volume (25 mL). Supernatants from the two centrifugations were pooled and an equal volume of saturated solution of $(NH_4)_2SO_4$ was added. The proteins were precipitated during stirring on ice for 40 min. The precipitate was collected by centrifugation at 16,000 *g* for 25 min and the pellet was resuspended in 20 mL PBS buffer containing 1 mM EDTA and 1 mM PMSF. After centrifugation at 100,000 *g* for 70 min at 4°, the 100,000 *g* supernatant was applied to an ATP-agarose column (Sigma A2767), and the column was eluted as described previously [27]. Purified 5-LO was immediately used for the 5-LO activity assay.

2.6. Determination of COX-2 product formation in MM6 cells

MM6 cells were grown with or without TGFβ and calcitriol for 96 hr as described [23]. Six hours prior harvest, LPS (100 ng/mL) was added. Cells were harvested, washed twice, resuspended in PGC buffer (5×10^6 cells/mL), and incubated with AA (30 μM) for 15 min at 37°. Cells were centrifuged (300 *g*, 5 min, 4°) and the amount of 6-keto $PGF_{1\alpha}$ released was assessed by ELISA using a monoclonal antibody against 6-keto $PGF_{1\alpha}$ [28] according to the protocol described by Yamamoto *et al*. [29]. For the ELISA, the monoclonal antibody (0.2 μg/200 μL) was coated to microtiter plates via a goat anti-mouse IgG antibody. 6-Keto $PGF_{1\alpha}$ (15 μg) was linked to bacterial β-galactosidase (0.5 mg, Calbiochem) and the enzyme activity bound to the antibody was determined in an ELISA reader at OD 550 nm (reference wavelength: 630 nm) using chlorophenol-red-β-D-galactopyranoside (CPRG, Roche Diagnostic GmbH) as substrate.

2.7. Western blot analysis

SDS–PAGE was performed using a mini Protean system (Bio-Rad) and 10% polyacrylamide gels. After electroblot to nitrocellulose membrane (Hybond C, Amersham), blocking with 5% non-fat dry milk in 50 mM Tris–HCl, pH 7.4, and 100 mM NaCl (TBS), membranes were washed and incubated with antibodies against COX-2 (1:1000 dilutions in TBS containing 2.5% (v/v) fetal calf serum) overnight at 4°. Immunoreactive COX-2 protein was visualized using alkaline phosphatase conjugated anti-rabbit IgG as described [26].

2.8. Statistics

The statistic program "GraphPad PRISM 3.0" was used for statistical comparisons. Calculations of IC_{50} values for compounds examined on inhibition of COX-1/2, and LO product formations are approximations.

3. Results

3.1. Effects of hyperforin and extracts of Hypericum perforatum on COX-1 activity in isolated human platelets

Freshly isolated human platelets were used to determine the effects of *H. perforatum* extracts and hyperforin

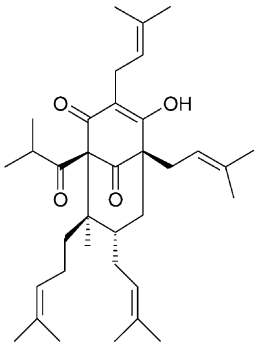

Fig. 1. Chemical structure of hyperforin.

(chemical structure, see Fig. 1) on COX-1 activity. As can be seen from Fig. 2A, hyperforin caused a dose-dependent suppression of the formation of the COX-1-derived product 12-HHT in intact platelets, stimulated with the physiological ligand thrombin ($IC_{50} = 0.3$ μM). In this respect, hyperforin was about 18-fold more potent than aspirin ($IC_{50} = 6$ μM), a common COX-1 inhibitor used as a positive control. To circumvent receptor-coupled cell activation, the Ca^{2+} ionophore A23187 (2 μM) was used to stimulate platelets for COX-1 product formation. As shown in Fig. 2B, hyperforin inhibited also the formation of 12-HHT in platelets challenged with A23187 ($IC_{50} = 3$ μM) being about 3-fold more potent than aspirin ($IC_{50} = 9$ μM). To exclude inhibitory effects of hyperforin on the endogenous substrate supply, platelets were stimulated with exogenous AA (20 μM) and COX-1 product formation was determined. Hyperforin suppressed 12-HHT formation also from exogenous AA with an IC_{50} of about 3 μM. The corresponding IC_{50} value for aspirin was 10 μM (Fig. 2C). Finally, potent COX-1 inhibition by hyperforin ($IC_{50} \approx 3$ μM) was found in platelet lysates, indicating that hyperforin might directly interfere with crude COX-1 activity (Fig. 2D). Similar inhibition of COX-1 product formation under all these conditions was found when equimolar amounts of two different standardized *H. perforatum* extracts containing 1.9 or 4% hyperforin, respectively, were used (data not shown). Neither aspirin nor hyperforin (up to 30 and 10 μM, respectively) caused significant change in 12-HETE formation in intact platelets (see the following description, Fig. 5B), indicating that the compounds do not affect platelet integrity or induce unspecific platelet inactivation. In summary, hyperforin is a potent inhibitor of COX-1 in intact platelets as well as in cell-free systems with a 3- to 18-fold higher efficacy than aspirin.

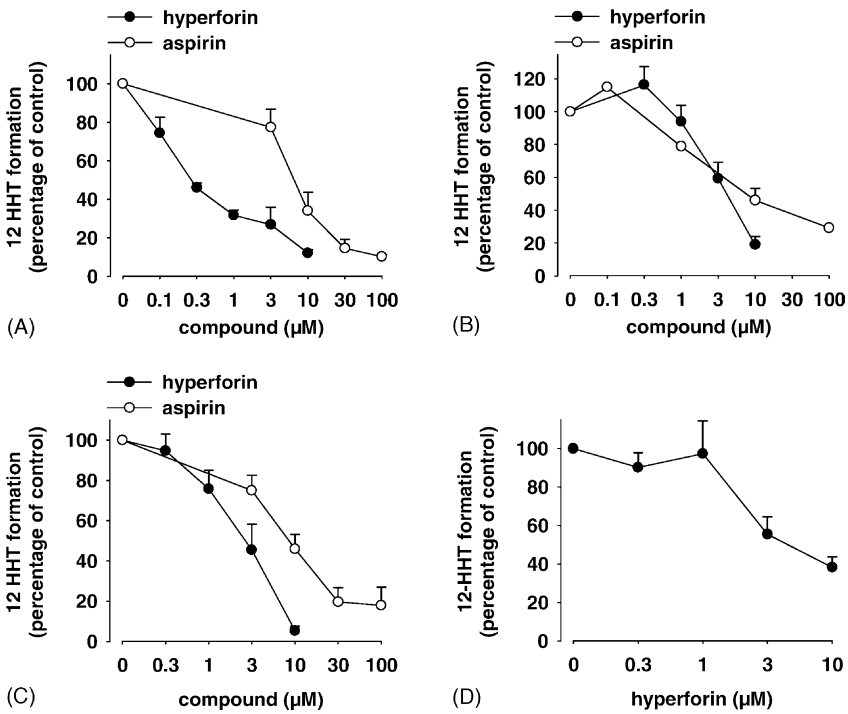

Fig. 2. Effects of hyperforin on COX-1 product formation in human platelets and corresponding homogenates. (A–C) Freshly isolated human platelets (1×10^8 cells/mL PGC buffer) were preincubated with the indicated concentrations of hyperforin or aspirin for 15 min at 37°. Then, cells were stimulated with (A) 5 U/mL thrombin, (B) 2 μM A23187, or (C) 20 μM exogenous AA, incubated for another 10 min at 37° and 12-HHT formation was determined by HPLC as described in the Section 2. The control values (100%) in absence of inhibitors were 50.1 ± 11.5, 37 ± 11.4, and 137.7 ± 26.7 ng/10^8 cells stimulated with thrombin, A23187, and AA, respectively. (D) Freshly isolated human platelets (1×10^8 cells/mL) were sonicated in PBS containing 1 mM EDTA on ice. Hyperforin was added, and after 5–10 min on ice samples were preincubated at 37° for 30 s. After addition of $CaCl_2$ and AA (2 mM and 20 μM, respectively) samples were incubated for another 10 min at 37° and 12-HHT formation was determined by HPLC as described. The control value (100%) in absence of inhibitors was 34.2 ± 26.7 ng/10^8 cells. Results are given as mean ± SE, N = 4.

3.2. Effects of hyperforin and Hypericum perforatum extracts on COX-2 activity

Inhibition of COX-2 was determined in LPS-stimulated MM6 cells that had been differentiated with TGFβ and calcitriol. It was shown that MM6 cells express high amounts of COX-2 upon stimulation with LPS in the absence of any detectable COX-1 protein and respond to sustained formation of PGs after addition of exogenous AA [30]. As shown from Fig. 3A, expression as well as product formation of COX-2 was further enhanced when MM6 cells had been differentiated with TGFβ and calcitriol prior stimulation with LPS. Consequently, possible inhibition of COX-2 by hyperforin was determined in TGFβ/calcitriol-differentiated MM6 cells after LPS treatment. As shown in Fig. 3B, hyperforin up to 30 μM had no inhibitory effect on 6-keto-$PGF_{1\alpha}$ formation in MM6 cells treated with 30 μM AA. Also, extracts of *H. perforatum* (corresponding to 30 μM hyperforin) did not suppress COX-2 activity (not shown). In control incubations, diclofenac (10 nM) potently suppressed COX-2 product formation under the same conditions. Thus, hyperforin inhibits COX-1 but seems unable to inhibit COX-2.

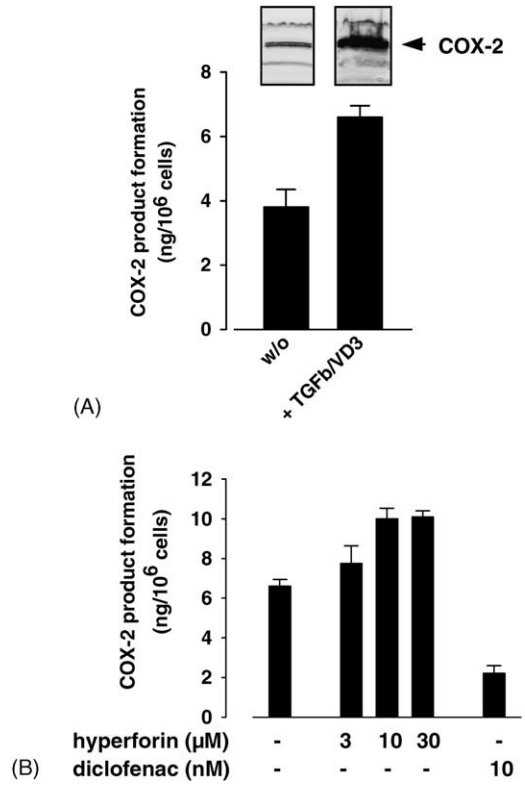

Fig. 3. Expression and product formation of COX-2 in MM6 cells; effect of hyperforin. (A) Expression and product formation of COX-2. MM6 cells were grown in presence or absence of TGFβ (1 ng/mL) and calcitriol (50 nM) for 96 hr, and LPS (100 ng/mL) was added 6 hr prior cell harvest. Aliquots corresponding to the same numbers of cells were analyzed for COX-2 protein by western blot (insert). To determine COX-2 product formation, the cells (4×10^6 per mL) were incubated with 30 μM AA for 15 min at 37°. The formed amounts of 6-keto $PGF_{1\alpha}$ were determined by ELISA as described. Results are given as mean ± SE, N = 4. (B) Inhibition of COX-2 product formation. MM6 cells, differentiated with TGFβ (1 ng/mL) and calcitriol (50 nM) for 96 hr and stimulated with LPS (100 ng/mL) for 6 hr prior harvest, were resuspended in PGC buffer and preincubated with the indicated additives for 15 min at 37°. Then, AA (30 μM) was added and after 10 min, the formation of 6-keto $PGF_{1\alpha}$ was determined by ELISA as described. The control value (100%) in absence of inhibitors was 6.6 ± 0.4 ng/10^6 cells. Results are given as mean ± SE, N = 4.

3.3. Inhibition of 5-LO product formation by hyperforin and Hypericum perforatum extracts in isolated human PMNLs

Freshly isolated PMNLs were incubated with hyperforin or extracts of *H. perforatum* and the formation of 5-LO products was determined. In PMNLs stimulated with A23187 (2.5 μM), hyperforin potently suppressed 5-LO product formation with an IC_{50} of approx. 1–1.5 μM (Fig. 4A). Similar efficient inhibition of 5-LO product formation by hyperforin was determined (IC_{50} approx. 1–2 μM), when cells had been challenged by A23187 in

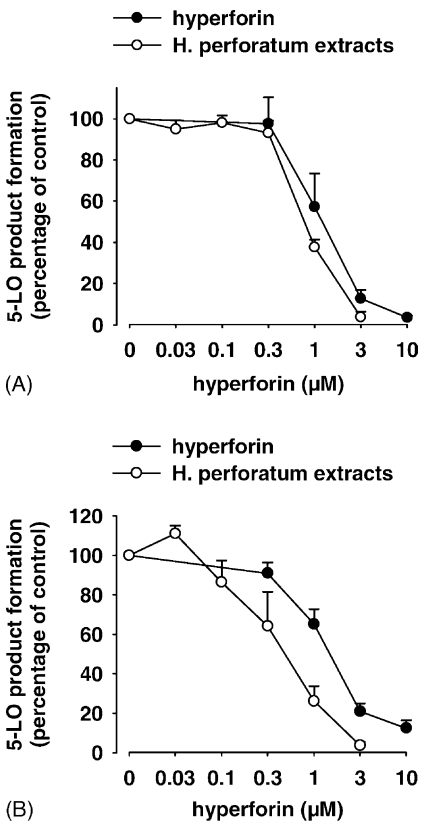

Fig. 4. Inhibition of 5-LO product formation by extracts of *Hypericum perforatum* and hyperforin in human isolated PMNLs. Freshly isolated human PMNLs (7.5×10^6 in 1 mL PGC buffer) were preincubated with the indicated amounts of *H. perforatum* extracts (calculated for hyperforin) or hyperforin for 15 min at 37°. After addition of 2.5 μM A23187 (A) or 2.5 μM A23187 plus 20 μM AA (B), samples were incubated for another 10 min and 5-LO products were determined by HPLC. Results are given as mean ± SE, N = 4–5. The control values (100%) in absence of inhibitors were 16.7 ± 2.1 and 144.6 ± 35.1 ng/10^6 cells, for cells stimulated in absence (A) and in presence (B) of AA, respectively.

presence of exogenous AA (20 μM), which was added to ensure sufficient substrate supply (Fig. 4B). Inhibition of 5-LO was somewhat more prominent for equimolar amounts of extracts of *H. perforatum* (calculated for hyperforin). Thus, the ED_{50} values were 22 μg/mL extract (containing 1.9% hyperforin) in absence of exogenous AA and 8 μg/mL in presence of 20 μM AA, which corresponds to ≈0.8 and ≈0.3 μM hyperforin, respectively.

To characterize the specificity of hyperforin for LO inhibition, we investigated the effects of hyperforin on 12-LO in platelets (see earlier description) and on 15-LO in PMNLs. PMNLs were treated as described for examination of 5-LO product formation and 15-HETE was measured to determine 15-LO inhibition. As can be seen from Fig. 5A, hyperforin showed poor inhibition of 15-LO product formation ($IC_{50} > 10$ μM). Also, hyperforin up to 10 μM

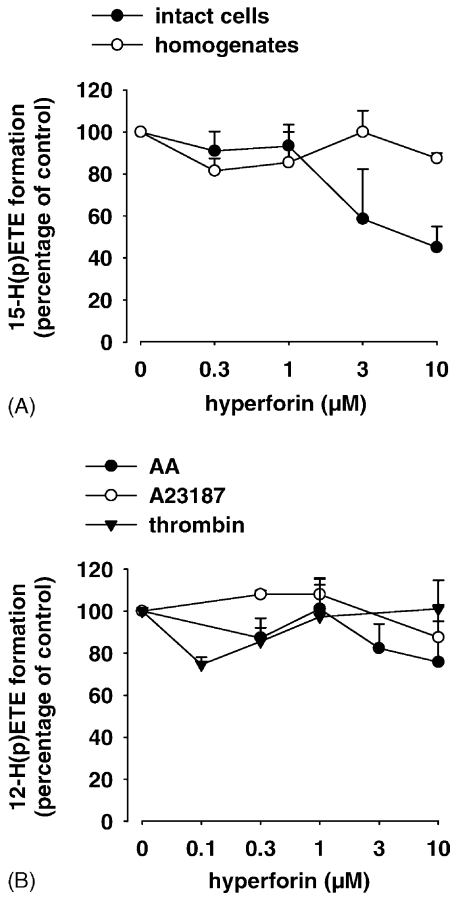

Fig. 5. Effects of hyperforin on product formation of 12- and 15-LO. (A) 15-LO in PMNLs. For determination of 15-LO product formation in intact cells, freshly isolated PMNLs (7.5×10^6 in 1 mL PGC buffer) were incubated with the indicated concentrations of hyperforin for 15 min at 37°. After addition of 2.5 μM ionophore plus 20 μM AA, samples were incubated for another 10 min and the formation of 15-HETE was determined by HPLC. For determination of 15-LO product formation in homogenates, PMNLs (7.5×10^6 in 1 mL PBS containing 1 mM EDTA) were sonicated, hyperforin was added and after 5–10 min, samples were prewarmed for 30 s at 37° and 20 μM AA was added. After 10 min at 37°, 15-LO product formation was determined by HPLC as described. Results are given as mean ± SE, N = 4. The control values (100%) in absence of inhibitors were 11.3 ± 5.4 and 20.1 ± 8.7 ng/10^6 cells, for intact cells and homogenates, respectively. (B) 12-LO in platelets. For determination of 12-LO product formation in intact cells, freshly isolated platelets (1×10^8 per mL) were incubated with the indicated concentrations of hyperforin for 15 min at 37°. After addition of the indicated stimuli, samples were incubated for another 10 min and the formation of 12-HETE was determined by HPLC. Results are given as mean ± SE, N = 4. The control values (100%) in absence of inhibitors were 2684 ± 405, 235 ± 43, and 213 ± 56 ng/10^8 cells, for cells stimulated with AA, A23187, and thrombin, respectively.

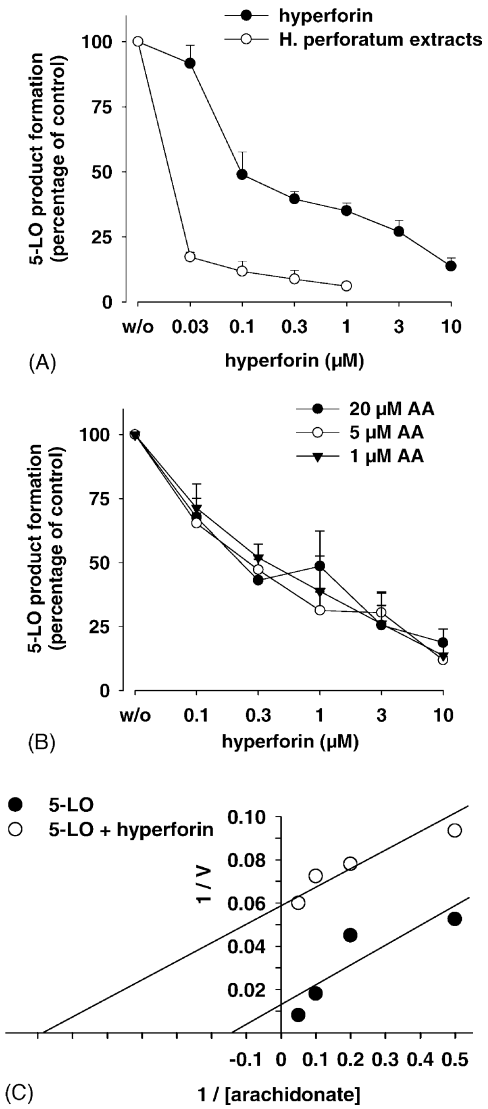

Fig. 6. Inhibition of product formation of purified 5-LO by *Hypericum perforatum* extracts and hyperforin; kinetic analysis. 5-LO, expressed in *Escherichia coli*, was purified as described. (A) Purified 5-LO (0.1 μg/mL) was preincubated with the indicated amounts of *H. perforatum* extracts or hyperforin for 5–10 min on ice. Samples were preincubated for 30 s at 37° and $CaCl_2$ and AA (2 mM and 20 μM, respectively) were added. After 10 min at 37°, 5-LO product formation was determined as described. Results are given as mean ± SE, N = 5–7. (B and C) Purified 5-LO (0.1 μg/mL) was preincubated with 1 μM hyperforin for 5–10 min on ice. Samples were preincubated for 30 s at 37° and 2 mM $CaCl_2$ and AA at the indicated concentrations were added. After 10 min at 37°, 5-LO product formation (B) was determined as described and kinetic analysis (C) of 5-LO inhibition (1 μM hyperforin) is given as Lineweaver–Burke plots. The AA concentrations were 1, 2.5, 5, and 10 μM.

could not suppress 12-HETE formation in platelets under all stimulation conditions (AA, A23187, or thrombin, Fig. 5B). Thus, hyperforin at submicromolar to low micromolar concentrations specifically interferes with the product formation of 5-LO, whereas other LOs (12- and 15-LO) are hardly affected.

To confirm a direct inhibitory effect of hyperforin on the 5-LO enzyme, human recombinant 5-LO was expressed in *E. coli*, purified to near homogeneity and 5-LO activity was determined in an optimized *in vitro* enzyme assay using 20 μM AA as substrate. Product formation of purified 5-LO was suppressed by hyperforin with an IC_{50} of approx. 90 nM (Fig. 6A). Notably, there was no complete suppression of 5-LO activity, even at high concentrations (up to 10 μM) of hyperforin. *Hypericum perforatum* extracts showed more than 10-fold higher efficacy for suppression of purified 5-LO enzyme activity than hyperforin alone (Fig. 6A).

In order to characterize the mode of action as a competitor for 5-LO, 5-LO inhibition (at 1 μM hyperforin) was determined at various substrate concentrations. As can be seen from Fig. 6B, 5-LO activity was potently suppressed regardless of the substrate concentrations (3–100 μM). Also, kinetic analyses demonstrate an uncompetitive 5-LO inhibition by hyperforin, as depicted from Lineweaver–Burke plots (Fig. 6C).

In contrast to hyperforin, the naphthodianthrone hypericin (up to 10 μM) did not block any enzyme activities (COX-1/2 and 5-,12-, and 15-LO) examined, neither in intact cells nor in cell lysates (data not shown). Taken together, hyperforin potently suppresses the biosynthesis of proinflammatory LTs and prostanoids by acting as a dual inhibitor of COX-1 and 5-LO in cell-free systems as well as in intact cells, without significant effects on related COX-2, and 12- and 15-LO.

4. Discussion

In this report we present novel molecular targets for hyperforin, namely COX-1 and 5-LO, key enzymes in eicosanoid biosynthesis. Hyperforin acts as a dual inhibitor on both enzymes with IC_{50} values for hyperforin in intact cells or in cell-free systems in the range of approx. 0.09–3 μM, respectively. These values are in a close range to the plasma concentration of hyperforin (up to 0.2–0.38 μM) after standard daily dosage of 3×300 mg *Hypericum* extract [31]. Of interest, the activities of the closely related oxygenases 12- and 15-LO as well as COX-2 appear to be not significantly affected by hyperforin, implying specificity for 5-LO and COX-1. Compared to the well-documented NSAID aspirin, hyperforin was found to be 3- to 18-fold more potent in COX-1 inhibition and it is almost equipotent to zileuton in inhibition of 5-LO (IC_{50} zileuton = 0.5–1 μM [32,33]).

Extracts of *H. perforatum* are currently used to treat mild to moderate depressive disorders [1–3]. *Hypericum* extracts contain at least 10 compounds that have been demonstrated to possess biological activity [34]. Recent evidence indicates that hyperforin (Fig. 1), the major lipophilic constituent of *H. perforatum*, is mainly responsible for the antidepressant activity [5,6,8]. Hyperforin has been shown to inhibit the re-uptake of several neurotransmitters and clinical effects of *H. perforatum* extracts on depression correlate with its hyperforin content [5,6,8]. Today, hyperforin is assumed as one of the main active ingredients of *H. perforatum*.

Aside of its use as antidepressive medicine, *H. perforatum* has been traditionally used in folk medicine for the topical treatment of inflammatory skin diseases, burns, and superficial wounds (see Ref. [12] and references therein), but also for the treatment of bronchitis, asthma, and rheumatoid arthritis. Notably, LTs and prostanoids which play a major role in the initiation and maintenance of inflammatory processes are connected to such disorders, suggesting that suppression of COX-1 and 5-LO by hyperforin may account for the beneficial effects of *H. perforatum* observed in traditional medicine. Of interest, 5-LO is expressed in central nervous system neurons and may participate in neurodegeneration [35], implying neuroprotective effects of hyperforin in aging brain. Moreover, recent studies showed that hyperforin possesses antibacterial activity [9,10], and in a panel of tumor cells but also in peripheral blood mononuclear cells, hyperforin inhibited proliferation and induced apoptosis [11,12]. Thus, hyperforin exerts important antiinflammatory properties that may act at multiple sites, inhibition of eicosanoid biosynthesis could be one important contribution.

In this study, we could rationalize these results by demonstrating for the first time that hyperforin inhibits eicosanoid formation *in vitro* and in intact human cells, implying that hyperforin directly acts on 5-LO and COX-1 and is available in the cell. For 5-LO, the IC_{50} values for purified enzyme (≈90 nM) and for intact cells (1–1.5 μM) differed by about 1 order of magnitude, which is generally observed for direct 5-LO inhibitors [16]. It is noteworthy to mention that the activities of the closely related 12- and 15-LO as well as COX-2 were not significantly suppressed by hyperforin, neither in intact cells nor in cell-free systems, suggesting a specific interaction of hyperforin with 5-LO and COX-1. Eicosanoid formation also from exogenous AA (20 μM) was blocked by hyperforin in platelets and PMNLs without a significant loss of efficacy. Thus, hyperforin does not simply suppress cellular eicosanoid formation by limiting endogenous AA supply via inhibition of cytosolic phospholipase A_2. For the activity of purified 5-LO, a difference between the efficacy of hyperforin and *H. perforatum* extracts was observed, which was less pronounced when cellular 5-LO product formation was determined. Thus, additional factor(s) in *H. perforatum* extracts (not cell-permeable and/or not active in intact cells) potentiate inhibition of 5-LO *in vitro*. Identification of these factor(s) remains a future task.

Compared to aspirin, hyperforin was about 3- to 18-fold more potent in inhibiting COX-1 in platelets. Particularly when platelets were stimulated with thrombin, COX-1 product formation was potently suppressed by hyperforin ($IC_{50} \approx 0.3$ μM). Importantly, at the same time hyperforin caused no significant change in the formation of 12-HETE, indicating that hyperforin does not generally interfere with thrombin-mediated signaling or release of endogenous AA, and does not lead to an compensatory increase in the formation of other eicosanoids. Moreover, COX-1 activity was inhibited by hyperforin also in cell-free systems, implying a direct enzyme interaction. In analogy to aspirin, hyperforin or extracts of *H. perforatum* may exert antithrombotic effects due to inhibition of thromboxane biosynthesis [36], although no such effects have been reported so far. Taken together, the naturally occurring hyperforin in *H. perforatum* efficiently suppresses prostanoid and LT biosynthesis *in vitro* as well as in intact cells, representing a dual inhibitor of COX-1 and 5-LO.

Data obtained from 5-LO-deficient mice as well as anti-LT therapy confirm the involvement of LTs in the pathogenesis of asthma and inflammatory diseases [16]. 5-LO has been the main target for pharmacological intervention with LT synthesis, although many most promising 5-LO inhibitors failed to enter the market due to inefficacy and/or severe side effects. Thus far zileuton is the only 5-LO inhibitor approved for the treatment of asthma [16]. According to their mode of action, 5-LO inhibitors can be classified either as redox-based or iron-ligand inhibitors (which act on the 5-LO active site iron), or as competitive nonredox-type 5-LO inhibitors [16]. Kinetic analyses using purified 5-LO reveal that hyperforin does not seem to act in a competitive manner at the 5-LO substrate binding site. Similarly, consistent COX-1 inhibition at various AA concentrations (not shown) revealed no competitive inhibition of the COX-1 enzyme. Also, due to the structural features, hyperforin should not possess chelating properties that cause 5-LO inhibition by interference with the active site iron. Moreover, hyperforin appears to be devoid of reducing properties and it failed to scavenge hydroperoxides in activated PMNLs (unpublished data). Therefore, hyperforin might possess an unique mode of action to suppress 5-LO activity that is not immediately apparent.

NSAIDs are the main drugs used to reduce the untoward consequences of inflammation by inhibition of COX enzymes [19], and have been proven beneficial in osteoarthritis and rheumatoid arthritis [18,37]. However, NSAIDs cause several serious adverse effects like gastric injury and ulceration, renal damage, and bronchospasm. Since selective inhibition of the inducible COX-2 should not affect renal function and gastric mucosa, selective COX-2 inhibitors have been developed [20,21]. In fact such compounds exert less gastric adverse effects compared to unspecific COX-1/2 inhibitors [38]. Nevertheless, recent data suggest that prostanoids formed by both COX enzymes might be involved in the inflammatory response and that also COX-2 may have physiological housekeeping functions (for review see Ref. [21]). For example, COX-1-deficient mice (that are perfectly healthy) showed a decreased ear inflammatory response to AA [39] and COX-1 was shown to account for the majority of prostanoids produced by inflamed human bursal tissue [21]. In another study, a significant antiinflammatory effect by selective COX-2 inhibitors was only observed after administration of doses that also inhibited COX-1 [40].

The lack of safe NSAIDs and LT synthesis inhibitors with high efficacy led to the development of the new class of the dual COX/5-LO inhibitors. It was found that inhibitors of COX enzymes and of 5-LO in combination are more effective than either class of drug administered alone [41]. In fact, studies with dual inhibitors indicate a broad-range antiinflammatory effectiveness devoid of gastric toxicity (for review see Ref. [20]). Among such COX-1/5-LO inhibitors, ML-3000 (which has entered phase III trials in ostheoarthritis) was shown to suppress COX-1 and 5-LO in bovine and human platelets and granulocytes with IC_{50} values of about 0.2 μM [42]. Despite potent inhibition of COX-1, repeated oral administration of ML-3000 produced no statistically significant gastrointestinal damage [20].

With regard to the properties of dual COX/5-LO inhibitors, hyperforin might be a potential drug possessing antiinflammatory activities devoid of the most troublesome (gastric) side effects seen for specific COX and 5-LO inhibitors. Although extracts of *H. perforatum* appear to be well tolerated and no serious adverse events were detected in many clinical studies [1–3], in some cases such extracts have been shown to trigger photosensitivity [1] and to influence pharmacokinetic drug interactions [13,43]. Moreover, the instability of hyperforin may limit the therapeutic use. Further evaluation of hyperforin in functional animal models will be necessary to estimate the clinical relevance of hyperforin as a dual-acting antiinflammatory drug.

In summary, hyperforin represents one of the most potent naturally occurring COX/5-LO inhibitors reported so far. Inhibition of COX-1 and 5-LO might explain the therapeutic indications of *H. perforatum* and hyperforin for the topical treatment of inflammatory and allergic skin diseases and suggest potential for the treatment of other allergic and inflammatory disorders.

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References

[1] Gaster B, Holroyd J. St. John's wort for depression: a systematic review. Arch Intern Med 2000;160(2):152–6.

[2] Greeson JM, Sanford B, Monti DA. St. John's wort (*Hypericum perforatum*): a review of the current pharmacological, toxicological, and clinical literature. Psychopharmacology (Berl) 2001;153(4):402–14.

[3] Di Carlo G, Borrelli F, Ernst E, Izzo AA. St. John's wort: Prozac from the plant kingdom. Trends Pharmacol Sci 2001;22(6):292–7.

[4] Linde K, Ramirez G, Mulrow CD, Pauls A, Weidenhammer W, Melchart D. St. John's wort for depression—an overview and meta-analysis of randomised clinical trials. Br J Med 1996;313(7052):253–8.

[5] Laakmann G, Schule C, Baghai T, Kieser M. St. John's wort in mild to moderate depression: the relevance of hyperforin for the clinical efficacy. Pharmacopsychiatry 1998;31(Suppl 1):54–9.

[6] Chatterjee SS, Bhattacharya SK, Wonnemann M, Singer A, Muller WE. Hyperforin as a possible antidepressant component of hypericum extracts. Life Sci 1998;63(6):499–510.

[7] Jensen AG, Hansen SH, Nielsen EO. Adhyperforin as a contributor to the effect of *Hypericum perforatum* L. in biochemical models of antidepressant activity. Life Sci 2001;68(14):1593–605.

[8] Singer A, Wonnemann M, Muller WE. Hyperforin, a major antidepressant constituent of St. John's wort, inhibits serotonin uptake by elevating free intracellular Na^+. J Pharmacol Exp Ther 1999;290(3): 1363–8.

[9] Schempp CM, Pelz K, Wittmer A, Schopf E, Simon JC. Antibacterial activity of hyperforin from St. John's wort, against multiresistant *Staphylococcus aureus* and Gram-positive bacteria. Lancet 1999; 353(9170):2129.

[10] Fiebich BL, Heinrich M, Langosch JM, Kammerer N, Lieb K. Antibacterial activity of hyperforin from St. John's wort. Lancet 1999;354(9180):777.

[11] Simon J, Schempp C, Schoepk E, Simon-Haarhaus B. Hyperforin as cytostatic agent and hyperforin ointment or cream as application form. PCT Int Appl 2000;39.

[12] Schempp CM, Winghofer B, Ludtke R, Simon-Haarhaus B, Schopf E, Simon JC. Topical application of St. John's wort (*Hypericum perforatum* L.) and of its metabolite hyperforin inhibits the allostimulatory capacity of epidermal cells. Br J Dermatol 2000;142(5):979–84.

[13] Moore LB, Goodwin B, Jones SA, Wisely GB, Serabjit-Singh CJ, Willson TM, Collins JL, Kliewer SA. St. John's wort induces hepatic drug metabolism through activation of the pregnane X receptor. Proc Natl Acad Sci USA 2000;97(13):7500–2.

[14] Otto JC, Smith WL. Prostaglandin endoperoxide synthases-1 and -2. J Lipid Mediators Cell Signal 1995;12(2/3):139–56.

[15] Samuelsson B, Dahlén S-E, Lindgren J-Å, Rouzer CA, Serhan CN. Leukotrienes and lipoxins: structures, biosynthesis, and biological effects. Science 1987;237:1171–6.

[16] Steinhilber D. 5-Lipoxygenase: a target for antiinflammatory drugs revisited. Curr Med Chem 1999;6:69–83.

[17] Parente L. Pros and cons of selective inhibition of cyclooxygenase-2 versus dual lipoxygenase/cyclooxygenase inhibition: is two better than one? J Rheumatol 2001;28(11):2375–82.

[18] Feldman M, McMahon AT. Do cyclooxygenase-2 inhibitors provide benefits similar to those of traditional nonsteroidal anti-inflammatory drugs, with less gastrointestinal toxicity? Ann Intern Med 2000; 132(2):134–43.

[19] Vane JR, Botting RM. Mechanism of action of anti-inflammatory drugs. Scand J Rheumatol Suppl 1996;102:9–21.

[20] Celotti F, Laufer S. Anti-inflammatory drugs: new multitarget compounds to face an old problem. The dual inhibition concept. Pharmacol Res 2001;43(5):429–36.

[21] Bertolini A, Ottani A, Sandrini M. Dual acting anti-inflammatory drugs: a reappraisal. Pharmacol Res 2001;44(6):437–50.

[22] Werz O, Brungs M, Steinhilber D. Purification of transforming growth factor beta1 from human platelets. Pharmazie 1996;51(11):893–6.

[23] Brungs M, Rådmark O, Samuelsson B, Steinhilber D. Sequential induction of 5-lipoxygenase gene expression and activity in Mono Mac 6 cells by transforming growth factor-beta and 1,25-dihydroxyvitamin D3. Proc Natl Acad Sci USA 1995;92(1):107–11.

[24] Böyum A. Isolation of mononuclear cells and granulocytes from human blood. Scand J Clin Lab Invest 1968;97(Suppl 21):77–89.

[25] Werz O, Steinhilber D. Selenium-dependent peroxidases suppress 5-lipoxygenase activity in B-lymphocytes and immature myeloid cells—the presence of peroxidase-insensitive 5-lipoxygenase activity in differentiated myeloid cells. Eur J Biochem 1996;242(1):90–7.

[26] Hammarberg T, Zhang YY, Lind B, Rådmark O, Samuelsson B. Mutations at the C-terminal isoleucine and other potential iron ligands of 5-lipoxygenase. Eur J Biochem 1995;230(2):401–7.

[27] Werz O, Szellas D, Henseler M, Steinhilber D. Nonredox 5-lipoxygenase inhibitors require glutathione peroxidase for efficient inhibition of 5-lipoxygenase activity. Mol Pharmacol 1998;54:445–51.

[28] Brune K, Reinke M, Lanz R, Peskar BA. Monoclonal antibodies against E- and F-type prostaglandins. High specificity and sensitivity in conventional radioimmunoassays. FEBS Lett 1985;186(1):46–50.

[29] Yamamoto S, Yokota K, Tonai T, Shono F, Hayashi Y. Enzyme immunoassay. Prostaglandins and related substances—a practical approach. Oxford: IRL Press; 1987.

[30] Berg J, Christoph T, Widerna M, Bodenteich A. Isoenzyme-specific cyclooxygenase inhibitors: a whole cell assay system using the human erythroleukemic cell line HEL and the human monocytic cell line Mono Mac 6. J Pharmacol Toxicol Methods 1997;37(4):179–86.

[31] Biber A, Fischer H, Romer A, Chatterjee SS. Oral bioavailability of hyperforin from *Hypericum* extracts in rats and human volunteers. Pharmacopsychiatry 1998;31(Suppl 1):36–43.

[32] Bell RL, Lanni C, Malo PE, Brooks DW, Stewart AO, Hansen R, Rubin P, Carter GW. Preclinical and clinical activity of zileuton and A-78773. Ann NY Acad Sci 1993;696:205–15.

[33] Carter GW, Young PR, Albert DH, Bouska J, Dyer R, Bell RL, Summers JB, Brooks DW. 5-Lipoxygenase inhibitory activity of zileuton. J Pharmacol Exp Ther 1991;256(3):929–37.

[34] Nahrstedt A, Butterweck V. Biologically active and other chemical constituents of the herb of *Hypericum perforatum* L. Pharmacopsychiatry 1997;30(Suppl 2):129–34.

[35] Manev H, Uz T, Sugaya K, Qu T. Putative role of neuronal 5-lipoxygenase in an aging brain. FASEB J 2000;14(10):1464–9.

[36] Schror K. Aspirin and platelets: the antiplatelet action of aspirin and its role in thrombosis treatment and prophylaxis. Semin Thromb Hemost 1997;23(4):349–56.

[37] DeWitt DL. Cox-2-selective inhibitors: the new super aspirins. Mol Pharmacol 1999;55(4):625–31.

[38] Masferrer JL, Zweifel BS, Manning PT, Hauser SD, Leahy KM, Smith WG, Isakson PC, Seibert K. Selective inhibition of inducible cyclooxygenase 2 in vivo is antiinflammatory and nonulcerogenic. Proc Natl Acad Sci USA 1994;91(8):3228–32.

[39] Langenbach R, Morham SG, Tiano HF, Loftin CD, Ghanayem BI, Chulada PC, Mahler JF, Lee CA, Goulding EH, Kluckman KD, et al. Prostaglandin synthase 1 gene disruption in mice reduces arachidonic acid-induced inflammation and indomethacin-induced gastric ulceration. Cell 1995;83(3):483–92.

[40] Wallace JL, Bak A, McKnight W, Asfaha S, Sharkey KA, MacNaughton WK. Cyclooxygenase 1 contributes to inflammatory responses in rats and mice: implications for gastrointestinal toxicity. Gastroenterology 1998;115(1):101–9.

[41] Nickerson-Nutter CL, Medvedeff ED. The effect of leukotriene synthesis inhibitors in models of acute and chronic inflammation. Arthritis Rheum 1996;39(3):515–21.

[42] Laufer S, Tries S, Augustin J, Dannhardt G. Pharmacological profile of a new pyrrolizine derivative inhibiting the enzymes cyclo-oxygenase and 5-lipoxygenase. Arzneimittelforschung 1994;44(5):629–36.

[43] Obach RS. Inhibition of human cytochrome P450 enzymes by constituents of St. John's wort, an herbal preparation used in the treatment of depression. J Pharmacol Exp Ther 2000;294(1):88–95.