British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01605.x
www.brjpharmacol.org

RESEARCH PAPER

On the inhibition of 5-lipoxygenase product formation by tryptanthrin: mechanistic studies and efficacy *in vivo*

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Keywords
tryptanthrin; 5-lipoxygenase; leukotriene; inflammation

Received
31 January 2011
Revised
23 June 2011
Accepted
14 July 2011

BACKGROUND AND PURPOSE

Leukotrienes (LTs) are pro-inflammatory mediators produced by 5-lipoxygenase (5-LO). Currently available 5-LO inhibitors either lack efficacy or are toxic and novel approaches are required to establish a successful anti-LT therapy. Here we provide a detailed evaluation of the effectiveness of the plant-derived alkaloid tryptanthrin as an inhibitor of LT biosynthesis.

EXPERIMENTAL APPROACH

We analysed LT formation and performed mechanistic studies in human neutrophils stimulated with pathophysiologically relevant stimuli (LPS and formyl peptide), as well as in cell-free assays (neutrophil homogenates or recombinant human 5-LO) and in human whole blood. The *in vivo* effectiveness of tryptanthrin was evaluated in the rat model of carrageenan-induced pleurisy.

KEY RESULTS

Tryptanthrin potently reduced LT-formation in human neutrophils (IC_{50} = 0.6 μM). However, tryptanthrin is not a redox-active compound and did not directly interfere with 5-LO activity in cell-free assays. Similarly, tryptanthrin did not inhibit the release of arachidonic acid, the activation of MAPKs, or the increase in $[Ca^{2+}]_i$, but it modified the subcellular localization of 5-LO. Moreover, tryptanthrin potently suppressed LT formation in human whole blood (IC_{50} = 10 μM) and reduced LTB_4 levels in the rat pleurisy model after a single oral dose of 10 mg·kg^{-1}.

CONCLUSIONS AND IMPLICATIONS

Our data reveal that tryptanthrin is a potent natural inhibitor of cellular LT biosynthesis with proven efficacy in whole blood and is effective *in vivo* after oral administration. Its unique pharmacological profile supports further analysis to exploit its pharmacological potential.

Abbreviations

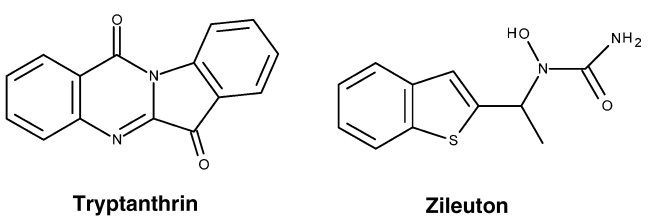
5-H(P)ETE, 5(S)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid; 5-LO, 5-lipoxygenase; AA, arachidonic acid; Ada, adenosine deaminase; $cPLA_2$, cytosolic phospholipase A_2; DMSO, dimethyl sulphoxide; DPPH, diphenylpicrylhydrazyl radical; DTT, dithiothreitol; FLAP, 5-lipoxygenase-activating protein; fMLP, N-formyl-methionyl-leucyl-phenylalanine; LT, leukotriene; PGC, phosphate-buffered saline plus 1 mg·mL^{-1} glucose and 1 mM $CaCl_2$; ROS, reactive oxygen species; SDS-b, SDS-PAGE sample loading buffer; STI, soybean trypsin inhibitor

Introduction

5-Lipoxygenase (5-LO) is a non-haeme iron-containing dioxygenase, which plays a central role in the biosynthesis of leukotrienes (LTs), a class of potent lipid mediators of inflammation and immunity (Peters-Golden and Henderson, 2007). 5-LO initiates LT biosynthesis by incorporation of molecular oxygen into arachidonic acid (AA) released by cytosolic phospholipase (cPL)A_2, followed by formation of the epoxide LT (LT)A_4. The unstable LTA_4 is further converted to other LTs (i.e. LTB_4 or cysteinyl-LTs) by LTA_4 hydrolase or LTC_4 synthase, depending on the cell type (Radmark *et al.*, 2007). 5-LO products have been established as mediators of asthma and allergic rhinitis, but may also have roles in sepsis, atherosclerosis, cardiovascular disease and cancer (Peters-Golden and Henderson, 2007). Accordingly, pharmacological suppression of 5-LO product synthesis is a major concept for intervention with LT-associated diseases (Werz and Steinhilber, 2006).

Intensive research in the field of LT synthesis inhibitors has led to the identification of different classes of compounds, acting directly on 5-LO enzymatic activity or on other enzymes or proteins involved in LT cellular biosynthesis [e.g. inhibitors of $cPLA_2$ or of 5-LO-activating protein (FLAP)]. Direct 5-LO inhibitors have been classified on the basis of their mechanism of action. Thus, iron ligand inhibitors chelate the active site iron, redox-active inhibitors uncouple the catalytic cycle of 5-LO, and non-redox-type 5-LO inhibitors compete with AA (Pergola and Werz, 2010). Most of these compounds, however, have failed in preclinical studies and/or in clinical trials due to their lack of efficacy or to toxicity. Until today, the iron ligand zileuton (Figure 1) was the only 5-LO inhibitor that could enter the market (Drazen *et al.*, 1999), but its clinical use is limited by liver toxicity.

Novel strategies for anti-LT intervention that may circumvent the problems associated with the typical classes of 5-LO inhibitors are therefore needed for steps forward in anti-LT therapy. With this aim, the plant kingdom represents a valuable source of structurally diverse compounds that might offer novel pharmacological concepts for interference with 5-LO (Werz, 2007; Feisst *et al.*, 2009). Although numerous plant-derived substances have been reported to interfere with LT synthesis, detailed investigations of 5-LO inhibition by natural compounds are rare (Werz, 2007). Most of these compounds are reducing agents like polyphenols (e.g. nordihydroguaiaretic acid), quinones or coumarins and seemingly act as redox-type 5-LO inhibitors with limited therapeutic value. Moreover, many analyses have often been conducted in only one type of assay (e.g. cell-free test model) and/or neglected pharmacologically relevant factors (i.e. plasma albumin-binding), which did not allow conclusions to be drawn regarding the pharmacological profile and therapeutic potential of the respective compounds.



Tryptanthrin Zileuton

Figure 1
Chemical structure of tryptanthrin and zileuton.

Tryptanthrin [indolo-(2,1-*b*)-quinazoline-6,12-dione] (Figure 1) is a weakly basic alkaloid, with unique structural features in comparison with other LT synthesis inhibitors, that occurs in various plant species such as *Isatis* (Danz *et al.*, 2001), *Strobilanthus* (Honda and Tabata, 1979), *Calanthe* (Murakami *et al.*, 2001) and *Couroupita* (Bergman *et al.*, 1985). Tryptanthrin has previously been shown to potently inhibit LTB_4 release in isolated granulocytes (IC_{50} = 0.15 μM) stimulated with ionophore (Danz *et al.*, 2002b). Based on these initial findings, tryptanthrin has generally been designated a 5-LO inhibitor (Danz *et al.*, 2002a; Lee *et al.*, 2007; Jao *et al.*, 2008), although its pharmacological profile is essentially unclear. Also, data on the efficacy of tryptanthrin on LT formation *in vivo* and corresponding anti-inflammatory effectiveness are lacking. Here we demonstrate the effectiveness of tryptanthrin in an animal model of disease related to LTs (i.e. carrageenan-induced pleurisy in the rat), and we provide a detailed evaluation of the effects of tryptanthrin on 5-LO product formation in diverse assay systems, taking into account essential parameters that may affect its effectiveness *in vivo*.

Methods

Materials

Tryptanthrin was synthesized and characterized as reported previously (Danz *et al.*, 2001). The $cPLA_{2\alpha}$ inhibitor N-{(2S,4R)-4-(biphenyl-2-ylmethyl-isobutyl-amino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl}-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl]acrylamide was from Calbiochem (Bad Soden, Germany). Zileuton was from Sequoia Research Products (Oxford, UK). Hyperforin and MK886 were generous gifts by Schwabe AG (Karlsruhe, Germany) and by Dr AW Ford-Hutchinson, Merck Frosst (Quebec, Canada), respectively. The λ-carrageenan type IV (isolated from *Gigartina aciculaire* and *Gigartina pistillata*) was from Sigma-Aldrich (Milan, Italy) as well as PGE_2 antibody. [^{3}H]-PGE_2 was from PerkinElmer Life Sciences (Milan, Italy). Antibodies recognizing phosphorylated extracellular signal-regulated protein kinases (pERK)1/2 (Thr202/Tyr204), phosphorylated p38 MAPK (Thr180/Tyr182), IκBα, NF-κB p65 protein, and phosphorylated proteins containing the phosphorylation motif of PKA (phospho-Ser/Thr residue with arginine at the –3 and –2 positions, RRXS/T) (Bryn *et al.*, 2006) were from Cell Signaling Technology (Danvers, MA, USA). HPLC solvents were from Merck (Darmstadt, Germany). All other chemicals were purchased from Sigma-Aldrich (Deisenhofen, Germany), unless stated otherwise.

Cells

Human neutrophils were freshly isolated from human venous blood of healthy adult donors (Blood Center, University Hospital, Tuebingen, Germany) as described previously (Werz *et al.*, 2002b). In brief, venous blood was taken from

donors and leucocyte concentrates were prepared by centrifugation at 4000× *g*, 20 min, 20°C. Neutrophils were immediately isolated by dextran sedimentation, centrifugation on Nycoprep cushions, and hypotonic lysis of erythrocytes. Neutrophils (purity > 96–97%) were finally resuspended in PBS pH 7.4 plus 1 mg·mL^{-1} glucose (PG buffer), or in PG buffer plus 1 mM $CaCl_2$ (PGC buffer) as indicated. For incubations with tryptanthrin, dimethyl sulphoxide (DMSO) was used as vehicle, never exceeding 0.1% (v v^{-1}). To exclude toxic effects of tryptanthrin during pre-incubation periods, neutrophil viability was analysed by light microscopy and trypan blue exclusion. Incubation with 30 μM tryptanthrin at 37°C for up to 30 min caused no significant change in neutrophil viability.

Expression and purification of human recombinant 5-LO from Escherichia coli

Human recombinant 5-LO was expressed in *E. 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 and lysed in 50 mM triethanolamine/HCl, pH 8.0, 5 mM EDTA, soybean trypsin inhibitor (STI; 60 μg·mL^{-1}), 1 mM PMSF, 1 mM dithiothreitol (DTT) and lysozyme (1 mg·mL^{-1}), homogenized by sonication (6 × 6 s) and centrifuged at 10 000× *g* for 15 min followed by centrifugation at 100 000× *g* for 70 min at 4°C. The supernatant was then applied to an ATP-agarose column (Sigma A2767; Sigma-Aldrich), and the column was eluted as described previously (Brungs *et al.*, 1995). Partially purified 5-LO was immediately used for *in vitro* activity assays.

Determination of 5-LO product formation

For assays of intact cells, freshly isolated neutrophils (3 × 10^7 mL^{-1}) were resuspended in 1 mL PGC buffer and primed at 37°C with LPS (1 μg·mL^{-1}) plus adenosine deaminase (Ada; 0.3 U·mL^{-1}) for 30 min. Formation of 5-LO products was started by addition of 1 μM formyl-methionyl-leucyl-phenylalanine (fMLP), and after 5 min the reaction was stopped with 1 mL of methanol and 30 μL of 1 N HCl, 200 ng PGB_1 and 500 μL of PBS were added. Test compounds or vehicle (0.1% DMSO) were added 15 min before fMLP. The samples were centrifuged (800× *g*, 10 min) and the supernatants were applied to C-18 solid-phase extraction columns (100 mg; UCT, Bristol, PA, USA) preconditioned with 1 mL methanol and 1 mL water. After being washed with 1 mL water and 1 mL 25% (v v^{-1}) methanol, formed 5-LO metabolites were eluted with 300 μL methanol. The eluates were diluted with 120 μL water, and 100 μL diluted extracts were analysed by reversed phase HPLC on a C-18 Radial-Pak® column (Waters, Eschborn, Germany) with 75% (v v^{-1}) methanol, 25% (v v^{-1}) water and 0.1% (v v^{-1}) trifluoroacetic acid as eluent (flow rate of 1.2 mL·min^{-1}) and detection at 280 and 235 nm. 5-LO products include LTB_4, its all-*trans* isomers, and 5(S)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid [5-H(P)ETE]. The chromatographic method used does not allow the measurement of cysteinyl-LTs (LTC_4, D_4 and E_4).

For determination of 5-LO product formation in neutrophil homogenates, 1 mM EDTA was added to cells resuspended in PBS. Samples were cooled on ice (5 min), sonicated (3 × 10 s) at 4°C, and 1 mM ATP and 1 mM DTT were added as indicated. For determination of human recombinant 5-LO activity, partially purified 5-LO was added to 1 mL of a 5-LO reaction mix (PBS, pH 7.4, 1 mM EDTA, 1 mM ATP). Samples of either partially purified 5-LO or of cell homogenates (corresponding to 10^7 neutrophils) were incubated for 10 min at 4°C with vehicle or tryptanthrin, pre-warmed for 30 s at 37°C and 2 mM $CaCl_2$ and the indicated concentrations of AA were added. The reaction was stopped after 10 min at 37°C by addition of 1 mL ice-cold methanol, and 30 μL of 1 N HCl, 200 ng PGB_1 and 500 μL of PBS were added. Formed metabolites were extracted and analysed by HPLC as described for intact neutrophils.

For assays in whole blood, freshly withdrawn blood from healthy adult donors was obtained by venipuncture and collected in monovettes containing 16 U heparin mL^{-1}. Aliquots of 2 mL (A23187) or 3 mL (LPS and fMLP) were pre-incubated with the test compounds or with vehicle (0.1% DMSO) for 15 min at 37°C, as indicated, and formation of 5-LO products was either started by addition of 1 μM fMLP following 30 min priming with 1 μg·mL^{-1} LPS, or by addition of 30 μM A23187. The reaction was stopped on ice after 15 (LPS and fMLP) or 10 (stimulation with A23187) min and the samples were centrifuged (600× *g*, 10 min, 4°C). Aliquots of the resulting plasma (500 μL) were then mixed with 2 mL of methanol and 200 ng PGB_1 were added as internal standard. The samples were placed at –20°C for 2 h and centrifuged again (600× *g*, 15 min, 4°C). The supernatants were collected and diluted with 2.5 mL PBS and 75 μL 1 N HCl. Formed 5-LO metabolites were extracted and analysed by HPLC as described for intact neutrophils.

Determination of release of ^{3}H-labelled AA from neutrophils

Release of ^{3}H-labelled AA from neutrophils was analysed as previously described (Fischer *et al.*, 2005). Briefly, freshly isolated neutrophils were resuspended at 2 × 10^6 in 1 mL RPMI 1640 medium containing 5 nM [^{3}H]-AA (corresponding to 1 μCi·mL^{-1}, specific activity 200 Ci·mmol^{-1}) and incubated for 120 min at 37°C in 5% CO_2 atmosphere. Cells were then collected by centrifugation, washed once with PBS and twice with PBS containing 2 mg·mL^{-1} fatty acid-free bovine albumin, to remove unincorporated [^{3}H]-AA. Labelled neutrophils (10^7) were resuspended in 1 mL PGC, primed with 1 μg·mL^{-1} LPS for 30 min and stimulated with 1 μM fMLP for 10 min at 37°C. Test compounds were added 15 min before fMLP. The samples were then placed on ice for 2 min and cells were centrifuged at 400 × *g* for 5 min at 4°C. Aliquots (100 μL) of the supernatants were measured by liquid scintillation in a beta-counter (Micro Beta Trilux, Perkin Elmer, Foster City, CA, USA) to detect the amounts of ^{3}H-labelled AA released into the medium.

Analysis of subcellular redistribution of 5-LO, IκBα and p65 by cell fractionation and immunoblotting

For analysis of 5-LO subcellular redistribution, neutrophils (3 × 10^7 mL^{-1} PGC buffer) were primed at 37°C with 1 μg·mL^{-1} LPS plus 0.3 U·mL^{-1} Ada for 30 min and stimulated by addition of 1 μM fMLP. After 5 min the reaction was stopped on ice and samples were centrifuged (200× *g* for 5 min at 4°C).

Tryptanthrin or vehicle (DMSO, 0.1%) was added 15 min before fMLP. IκBα and p65 subcellular redistribution was analysed as described (Vancurova *et al.*, 2001) with slight modifications. In brief, neutrophils (1×10^7 mL^{-1} phenol red-free RPMI medium supplemented with 10% low endotoxin fetal calf serum) were pre-incubated at 37°C for 10 min with vehicle (DMSO, 0.1%) or tryptanthrin and then 1 μg·mL^{-1} LPS or 1 μM fMLP were added for 30 min at 37°C. The reaction was stopped on ice, 1 mM PMSF was added and samples were centrifuged (600× *g* for 5 min at 4°C) and washed twice with ice-cold PBS containing 1 mM PMSF. Subcellular fractionation was performed by mild detergent (0.1% NP40) lysis, yielding a nuclear and a non-nuclear fraction (Werz *et al.*, 2001).

Briefly, neutrophils were suspended in ice-cold NP-40-lysis buffer (10 mM Tris/HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 0.1% NP-40, 1 mM PMSF, 60 μg·mL^{-1} STI, and 10 μg·mL^{-1} leupeptin), vortexed (3 × 5 s), kept on ice for 10 min and centrifuged (800× *g* for 10 min at 4°C). The resulting supernatants (non-nuclear fractions) were transferred to a new tube and the pellets (nuclear fractions) were resuspended in ice-cold relaxation buffer (50 mM Tris/HCl, pH 7.4, 250 mM sucrose, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF, 60 μg·mL^{-1} STI, 10 μg·mL^{-1} leupeptin). Both nuclear and non-nuclear fractions were centrifuged again (800× *g*, 10 min, 4°C), for further purification. Lysis of cells and integrity of nuclei was confirmed by light microscopy with trypan blue exclusion. Nuclei in relaxation buffer were disrupted by sonication (3 × 5 s).

Nuclear and non-nuclear fractions were immediately mixed with the same volume of SDS-PAGE sample loading buffer (SDS-b; 20 mM Tris/HCl, pH 8, 2 mM EDTA, 5% SDS (w v^{-1}), 10% β-mercaptoethanol) and heated for 6 min at 95°C. Aliquots (20 μL) of these samples were analysed by SDS-PAGE using a Mini Protean system (Bio-Rad, Hercules, CA, USA) on 10% gels. Correct loading of the gel and transfer of proteins to the nitrocellulose membrane was confirmed by Ponceau staining. After electroblotting to nitrocellulose membrane (GE Healthcare, Munich, Germany), membranes were blocked with 5% bovine serum albumin in 50 mM Tris/HCl, pH 7.4 and 100 mM NaCl (TBS) for 1 h at room temperature (RT). Membranes were washed and then incubated with primary antibody overnight at 4°C. 5-LO antiserum (1551, AK7, affinity purified on a 5-LO column, kindly provided by Dr Olof Rådmark, Karolinska Institute, Stockholm, Sweden) was used as 1:100 dilution. The membranes were washed, incubated with 1:1000 dilution of alkaline phosphatase-conjugated IgGs for 2 h at RT, and proteins were visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate in detection buffer (100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$).

Analysis of subcellular localization of 5-LO by indirect immunofluorescence microscopy

Neutrophils (1.5×10^6 in 500 μL PGC buffer) were primed at 37°C with LPS (1 μg·mL^{-1}) plus Ada (0.3 U·mL^{-1}). After 15 min, test compounds or vehicle (0.1% DMSO) were added and samples were incubated for additional 15 min at 37°C. Cells were then centrifuged at 10× *g* for 30 s onto poly-L-lysine (MW 150 000–300 000; Sigma-Aldrich)-coated glass coverslips, and activated by addition of 1 μM fMLP for 3 min at 37°C. Cells were fixed in methanol (–20°C, 30 min) and permeabilized with 0.1% Tween 20 in PBS (RT, 10 min), followed by three washing steps with PBS. Samples were blocked with 10% non-immune goat serum (Invitrogen, Darmstadt, Germany) for 10 min at RT, washed with PBS and incubated with anti-5-LO serum (1551, affinity purified) for 30 min at RT. The coverslips were washed ten times with PBS, incubated for 10 min at RT in the dark with Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) diluted 1 to 1500 in PBS, and washed 10 times with PBS. The coverslips were then mounted on glass slides with Mowiol (Calbiochem) containing 2.5% n-propyl gallate (Sigma-Aldrich). The fluorescence was visualized with a Zeiss Axio Observer.Z1 microscope and a Plan-Apochromat 100x/1.40 Oil DIC M27 objective (Carl Zeiss AG, Jena, Germany). An AxioCam MR3 camera was used for image acquisition.

Diphenylpicrylhydrazyl (DPPH) assay

The antioxidant activity of tryptanthrin was assessed by the method of Blois (Blois, 1958), with slight modifications. Briefly, 100 μL of 25, 50, 100 or 200 μM tryptanthrin in ethanol (corresponding to 2.5, 5, 10 or 20 nmol) were added to 100 μL of a solution of the stable free radical DPPH in ethanol (50 μM, corresponding to 5 nmol), buffered with acetate to pH 5.5, in a 96 well plate. The absorbance was recorded at 520 nm after 30 min incubation under gentle shaking in the dark. Ascorbic acid and L-cysteine were used as reference compounds. All analyses were performed in triplicates.

Evaluation of the phosphorylation state of MAPK and of proteins containing the PKA-phosphorylation motif

Neutrophils (10^7 in 100 μL PGC buffer) were preincubated with the indicated concentrations of tryptanthrin or vehicle for 15 min. Then, 1 μM fMLP was added for 1.5 min at 37°C. Alternatively, cells were directly incubated with tryptanthrin or vehicle for 1.5 min at 37°C. The reaction was stopped by addition of 100 μL of SDS-b, and heated for 6 min at 95°C. Proteins in cell lysates were separated by SDS-PAGE on a 10% gel, electroblotted on Hybond ECL nitrocellulose membranes (GE Healthcare) and analysed for phosphorylated ERK1/2, phosphorylated p38-MAPK and phosphorylated proteins containing the PKA phosphorylation motif (RRXS/T) using an Ettan-DIGE imaging system (GE Healthcare) as described (Pergola *et al.*, 2008).

Reactive oxygen species (ROS) generation assay

Neutrophils (10^7 mL^{-1} PG buffer) were preincubated with tryptanthrin or vehicle (0.1% DMSO) for 15 min. Then, the peroxide-sensitive fluorescence dye 2′,7′-dichlorofluorescein-diacetate (1 μg·mL^{-1}) and $CaCl_2$ (1 mM) were added 2 min before addition of 1 μM fMLP. The fluorescence emission at 530 nm was measured after excitation at 485 nm in a temperature-controlled (37°C) cuvette with continuous stirring in a spectrofluorometer (Aminco-Bowman series 2, Thermo Spectronic, Rochester, NY, USA).

Evaluation of intracellular Ca^{2+} mobilization

The determination of intracellular Ca^{2+} levels was performed as described previously (Werz *et al.*, 2002b). In brief, freshly

isolated neutrophils (10^7) were resuspended in 1 mL PG buffer and incubated with 2 μM Fura-2/AM for 30 min at 37°C. After washing, cells were finally resuspended in 1 mL PG buffer, preincubated with tryptanthrin or vehicle (0.1% DMSO) for 15 min, supplemented with 1 mM $CaCl_2$, and transferred into a temperature-controlled (37°C) fluorimeter cuvette in a spectrofluorometer (AB-2) with continuous stirring. Mobilization of intracellular Ca^{2+} was initiated by addition of fMLP (0.1 μM). The fluorescence emission at 510 nm was measured after excitation at 340 and 380 nm, respectively. Intracellular Ca^{2+} levels were calculated according to the method of Grynkiewicz *et al*. (1985). F_{max} (maximal fluorescence) was obtained by lysing the cells with 0.5% Triton-X 100 and F_{min} by chelating Ca^{2+} with 10 mM EDTA.

Carrageenan-induced pleurisy in rats

Male Wistar Han rats (220–230 g, Harlan, Udine, Italy) were housed in a controlled environment and provided with standard rodent chow and water. All animal care and experimental procedures complied 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). Tryptanthrin (10 $mg \cdot kg^{-1}$) or vehicle (0.5 mL of 0.5% carboxymethylcellulose and 10% Tween 20) was given p.o. 1 h before carrageenan. Rats were anaesthetized with enflurane 4% mixed with O_2, 0.5 $L \cdot min^{-1}$, N_2O 0.5 $L \cdot min^{-1}$ 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% λ-carrageenan type IV (w v^{-1}) (0.2 mL) was injected into the pleural cavity. The skin incision was closed with a suture, and the animals were allowed to recover. At 4 h after the injection of carrageenan, the animals were killed by inhalation of CO_2. The chest was carefully opened, and the pleural cavity was rinsed with 2 mL saline solution containing heparin (5 $U \cdot mL^{-1}$). The exudates and washing solution were removed by aspiration, and the total volume was measured. Any exudate that was contaminated with blood was discarded. Leucocytes in the exudates were resuspended in PBS and counted with an optical light microscope in a Burker's chamber after vital trypan blue staining. The amount of LTB_4 in the supernatant of centrifuged exudate (800× *g* for 10 min) was below the detection limit of the HPLC and was assayed by enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's protocol. The amount of PGE_2 in the supernatant of centrifuged exudate (800× *g* for 10 min) was assayed by radioimmunoassay. The results are expressed as ng per rat.

Statistics

Results are expressed as mean ± SEM of *n* observations, where *n* represents the number of experiments performed on different days in duplicates or the number of animals, as indicated. The IC_{50} values were determined by interpolation on semilogarithmic graphs and validated with GraphPad Instat program. Data fit was obtained using the sigmoidal dose-response equation (variable slope) (GraphPad Prism software, GraphPad, San Diego, CA, USA). Statistical evaluation of the data was performed by one-way ANOVA for independent or correlated samples followed by Tukey's HSD *post hoc* tests. Where appropriate, Student's *t*-test was applied. A P value < 0.05 (*) was considered significant.

Results

Tryptanthrin inhibits 5-LO product formation in intact neutrophils

Previous studies showed that tryptanthrin suppresses LTB_4 synthesis in neutrophils stimulated with the unphysiological stimulus A23187 ionophore (Danz *et al*., 2002b). Neutrophils are the major source of LTs in blood (Surette *et al*., 1993) and stimulation of LT synthesis with the natural agonists LPS and fMLP uses defined cellular pathways to activate 5-LO (i.e. Ca^{+2} and MAPK) (Surette *et al*., 1993; Werz *et al*., 2002a) and is considered to reflect pathophysiologically relevant conditions at inflammation sites. Tryptanthrin effectively suppressed 5-LO product formation in LPS and fMLP-stimulated neutrophils with an $IC_{50} = 0.6 \pm 0.2$ μM, and was essentially equipotent to the 5-LO reference inhibitor zileuton ($IC_{50} = 0.7 \pm 0.1$ μM) (Figure 2A). The formation of the 5-LO metabolites LTB_4 and 5-H(P)ETE was inhibited with a similar potency by tryptanthrin (not shown).The suppression of 5-LO product formation under the assay conditions described earlier could be due to inhibition of substrate supply, for example, by interference with AA release. Stimulation of human neutrophils with LPS and fMLP resulted in a 1.54-fold increase in AA release, which is in agreement with previous findings (Pergola *et al*., 2008). Tryptanthrin (up to 30 μM) did not significantly inhibit LPS and fMLP-induced increase in AA release, whereas a specific $cPLA_2$ inhibitor prevented it (Figure 2B).

Tryptanthrin is not a direct 5-LO inhibitor

A major unresolved question is whether inhibition of cellular LT formation by tryptanthrin is due to direct interference with 5-LO enzymatic activity. Most of the plant-derived 5-LO inhibitors possess reducing properties and act by reducing the active site iron of 5-LO (Werz, 2007). Therefore, we evaluated the redox potential of tryptanthrin and we analysed its radical scavenging properties in the well-recognized DPPH assay. Tryptanthrin did not reduce the DPPH radical, whereas L-cysteine and ascorbic acid acted as expected (Figure 3A).

We next analysed whether tryptanthrin inhibits the activity of crude 5-LO in cell-free systems. In neutrophil homogenates, 5-LO activity was not inhibited by tryptanthrin up to 30 μM and only 21% (non-significant) inhibition was observed at 100 μM (Figure 3B). An impaired efficacy of 5-LO inhibition in cell-free systems has also previously been observed for certain direct 5-LO inhibitors, which could be restored by varying the assay conditions. For instance, non-redox-type 5-LO inhibitors are less active in cell-free systems due to high peroxide levels, and inclusion of thiols restores their efficacy (Fischer *et al*., 2004). Also, the potency of hyperforin is strongly attenuated by cellular membranes present in cell homogenates, but is preserved when partially purified 5-LO is analysed (Feisst *et al*., 2009). Addition of exogenous DTT to neutrophil homogenates in order to decrease the lipid hydroperoxide levels did not significantly restore 5-LO inhibition by tryptanthrin (Figure 3B). In addition, tryptanthrin did not significantly inhibit partially purified human recom-

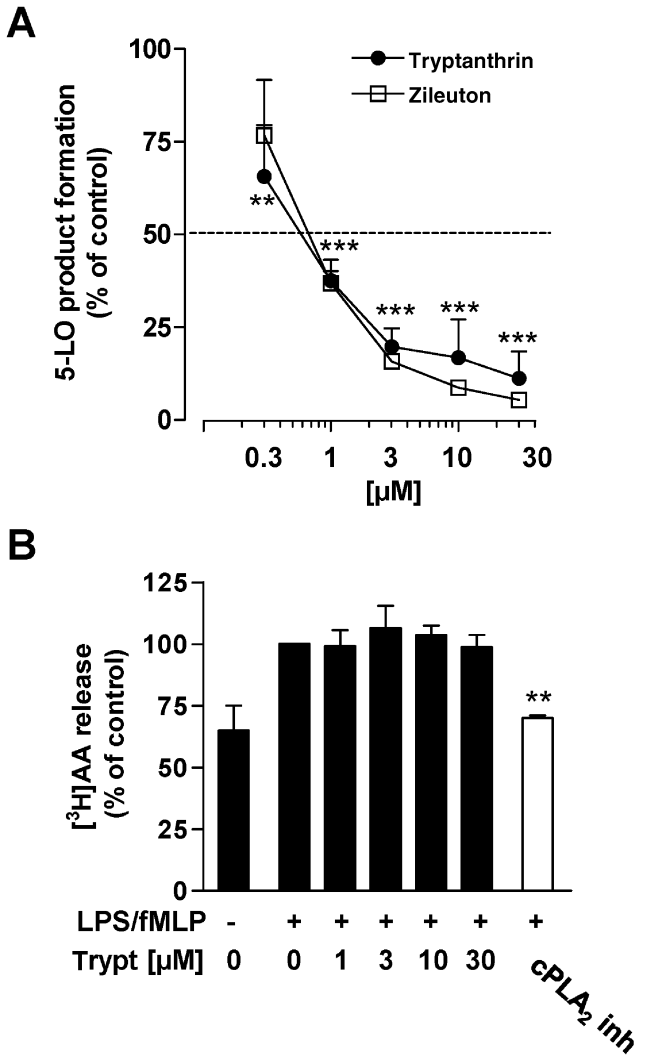


Figure 2

Tryptanthrin inhibits 5-LO product formation in intact neutrophils. Effect of (A) tryptanthrin or zileuton on 5-LO product synthesis in neutrophils stimulated with fMLP (1 μM; 5 min, 37°C) after 30 min priming with LPS (1 μg·mL^{-1}) plus Ada (0.3 U·mL^{-1}). Data are expressed as percentage of control, means + SEM; $n = 3$, duplicates. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; ANOVA + Tukey's test. 5-LO products in 100% controls were (ng 10^{-7} cells): 10.9 ± 3.6. (B) Effect of tryptanthrin and of the cPLA$_{2\alpha}$ inhibitor (15 μM) on the release of [^{3}H]-AA in intact neutrophils, following activation with fMLP (1 μM; 5 min, 37°C) after 30 min priming with LPS (1 μg·mL^{-1}). Data are expressed as percentage of control, means + SEM; $n = 3$, duplicates. $^{**}P < 0.01$ vs. control; ANOVA + Tukey's test.

binant 5-LO under standard assay conditions (20 μM AA as substrate, 1 mM ATP and 1 mM Ca^{2+} as supplements), whereas the direct 5-LO inhibitor zileuton (used as control) reduced 5-LO product formation by 79% at 3 μM (Figure 3C). Also, no significant direct inhibitory effects of tryptanthrin on 5-LO activity were observed after removal of Ca^{2+} as stimulating cofactor or by lowering the substrate concentration (from 20 to 2 μM AA, not shown).

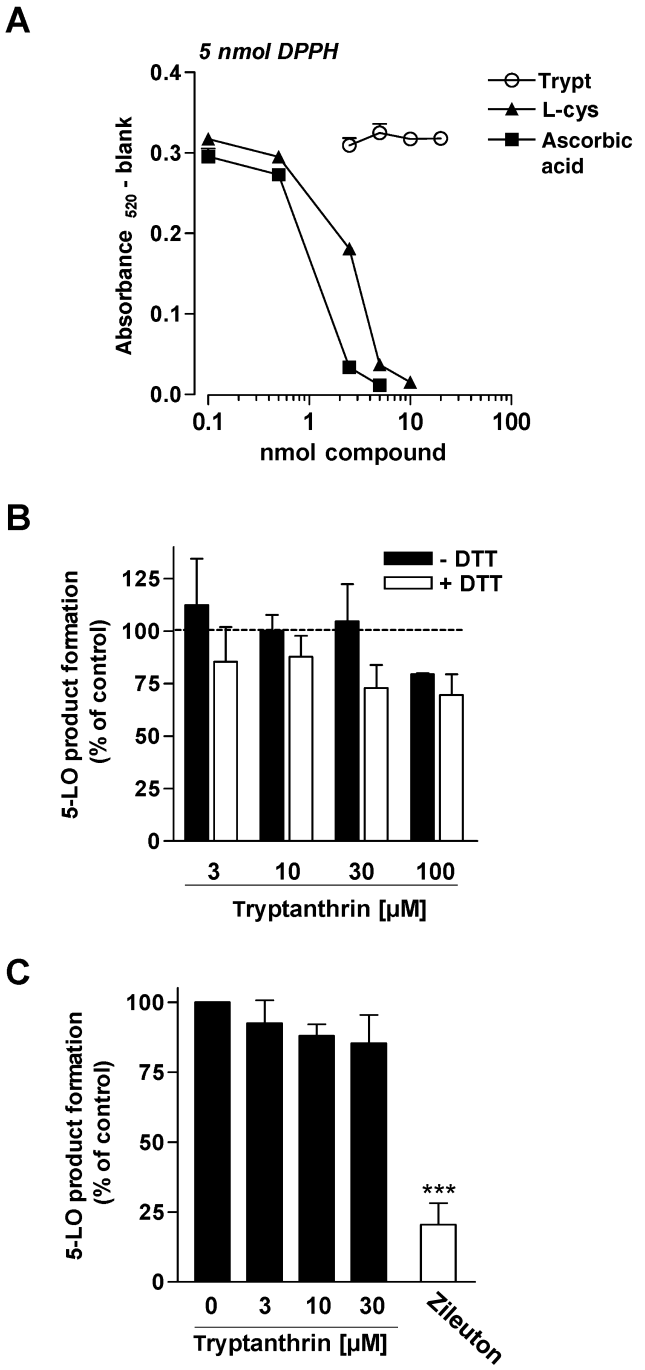


Figure 3

Tryptanthrin is not a direct 5-LO inhibitor. (A) Reduction of the DPPH radical by tryptanthrin, L-cysteine and ascorbic acid. Data are means + SEM; triplicates. (B) Effect of tryptanthrin on 5-LO activity in neutrophil homogenates (20 μM AA, 1 mM free Ca^{+2}, 1 mM ATP; ±1 mM DTT, as indicated). Data are expressed as percentage of control, means + SEM; $n = 3$, duplicates. 5-LO products in 100% controls were (ng mL^{-1}): without DTT, 522.2 ± 104.0, plus DTT: 990.9 ± 63.5. (C) Effect of tryptanthrin at the indicated concentrations and of 3 μM zileuton on the activity of partially purified 5-LO (20 μM AA, 1 mM free Ca^{+2}, 1 mM ATP). Data are expressed as percentage of control, means + SEM; $n = 3$, duplicates. $^{***}P < 0.001$ vs. control; ANOVA + Tukey's test. 5-LO products in 100% controls were (ng mL^{-1}): 1312.0 ± 237.5.

Analysis of the effect of tryptanthrin on 5-LO subcellular localization

Based on the finding that despite potently suppressing cellular 5-LO product formation, tryptanthrin failed to directly inhibit 5-LO, we investigated possible points of attack of tryptanthrin, which may cause suppression of 5-LO product synthesis in intact cells. A major event governing cellular 5-LO product formation is the localization of 5-LO at the nuclear membrane and access to its substrate (Werz *et al.*, 2001; Werz, 2002). Stimulation of neutrophils by LPS and fMLP caused redistribution of 5-LO from the non-nuclear to the nuclear compartment, as assessed by subcellular fractionation by mild-detergent lysis and 5-LO immunodetection (Figure 4A). Tryptanthrin did not reduce LPS and fMLP-induced 5-LO translocation to the nucleus. Also the FLAP inhibitor MK886 only partially increased the amount of 5-LO in the non-nuclear fraction under these conditions. We therefore further analysed 5-LO subcellular localization by immunofluorescence microscopy (Figure 4B). In resting cells, (a) 5-LO was homogeneously distributed in the cytosol. Interestingly, after incubation with tryptanthrin we observed that (b) 5-LO accumulated within the perinuclear region. After activation with LPS and fMLP, (c) 5-LO was localized at the nuclear membrane, as indicated by the stain tracing out the three-lobulated nuclei of neutrophils, which was only partially altered by tryptanthrin (d) and both nuclear membrane and perinuclear staining were evident. The FLAP inhibitor MK886 (e) prevented the nuclear membrane localization of 5-LO induced by LPS and fMLP but did not restore the homogeneous cytosolic staining of 5-LO observed in resting cells. The compound interfering with the C2-like domain of 5-LO hyperforin (f) also reduced the amount of 5-LO at the nuclear membrane.

Analysis of the effect of tryptanthrin on mechanisms governing cellular 5-LO product formation and on NF-κB activation

Cellular activation of 5-LO activity is also regulated by crucial signalling molecules (e.g. MAPKs, Ca^{2+}, PKA) and by cellular peroxides (Werz and Steinhilber, 2005). Incubation of human neutrophils with tryptanthrin (30 μM) partially increased the phosphorylation state of p38 MAPK, but did not alter the phosphorylation of ERK1/2 and of PKA substrate proteins containing the phosphorylation motif RRXS/T (Figure 5A,B). However, tryptanthrin (1 to 30 μM) did not influence MAPK phosphorylation induced by fMLP, and also 30 μM tryptanthrin failed to suppress fMLP-stimulated increase in $[Ca^{2+}]_i$ (Figure 5C,D). Similarly, fMLP-induced elevation of the cellular peroxide tone was not reduced by tryptanthrin (30 μM, Figure 5E). In previous studies addressing the effects of tryptanthrin on cytokine production by lymphocytes, the inhibition of NF-κB has been hypothesized as a possible mechanism of action (Takei *et al.*, 2003). However, tryptanthrin (30 μM) did not prevent LPS-induced degradation of IκBα in the cytosolic and nuclear fractions of neutrophils [an index of activation of NF-κB in neutrophils (Vancurova *et al.*, 2001)] and/or the LPS-induced increase of p65 in the nucleus (Figure 5F,G).

Tryptanthrin inhibits 5-LO product formation in human whole blood

Despite their strong efficacy in isolated cells, several inhibitors of LT biosynthesis failed to reduce 5-LO product formation in whole blood assays, seemingly due to high binding to plasma albumin or to interference/competition with other blood cells or with blood components (e.g. fatty acids) (Werz, 2002). Accordingly, these compounds also failed in subsequent *in vivo* studies in terms of both inhibition of LT syn-

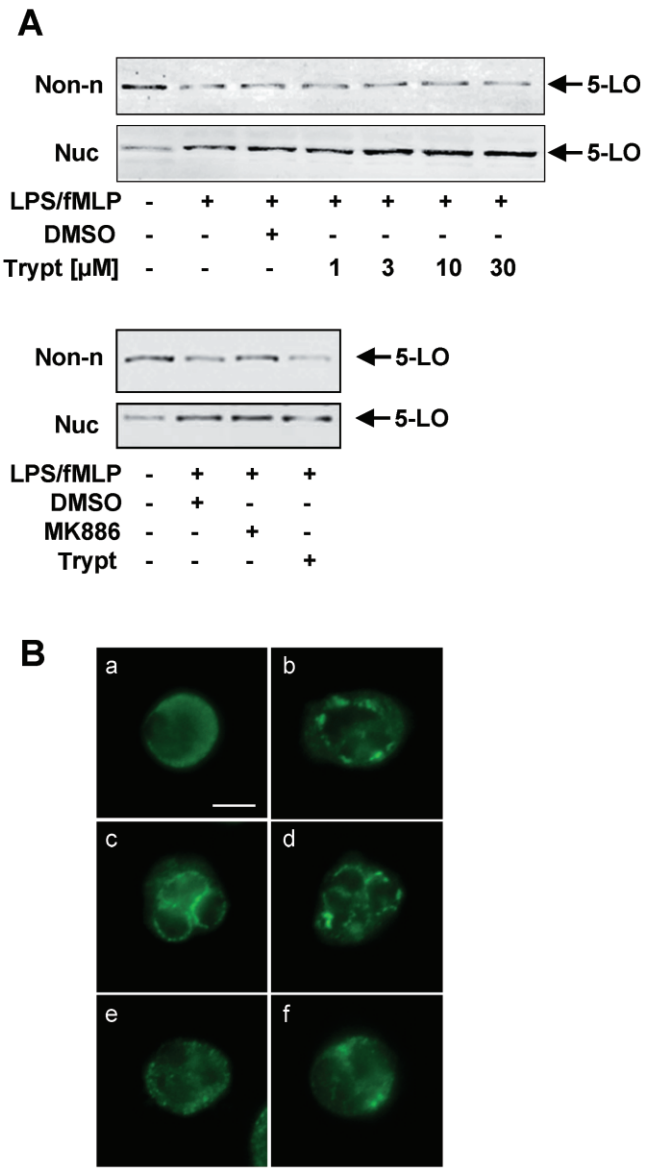


Figure 4

Effect of tryptanthrin on 5-LO subcellular localization in human neutrophils. Effect of tryptanthrin and MK886 (100 nM) on 5-LO subcellular localization following activation of neutrophils with fMLP (1 μM; 5 min, 37°C) after 30 min priming with LPS (1 $\mu g \cdot mL^{-1}$) plus Ada (0.3 $U \cdot mL^{-1}$), analysed by (A) immunodetection of 5-LO in the nuclear (Nuc) and non-nuclear (Non-n) fractions of mild-detergent (0.1% NP-40)-lysed cells and (B) by immunofluorescence microscopy. (a) Vehicle; (b) tryptanthrin (30 μM); (c) vehicle (0.1% DMSO) + LPS/Ada/fMLP; (d) tryptanthrin (30 μM) + LPS/Ada/fMLP; (e) MK886 (100 nM) + LPS/Ada/fMLP; (f) hyperforin (10 μM) + LPS/Ada/fMLP. The results shown are representative of 3 independent experiments. In (B) pictures with staining for 5-LO (green, Alexa Fluor 488) are shown; scale bar, 5 μm.

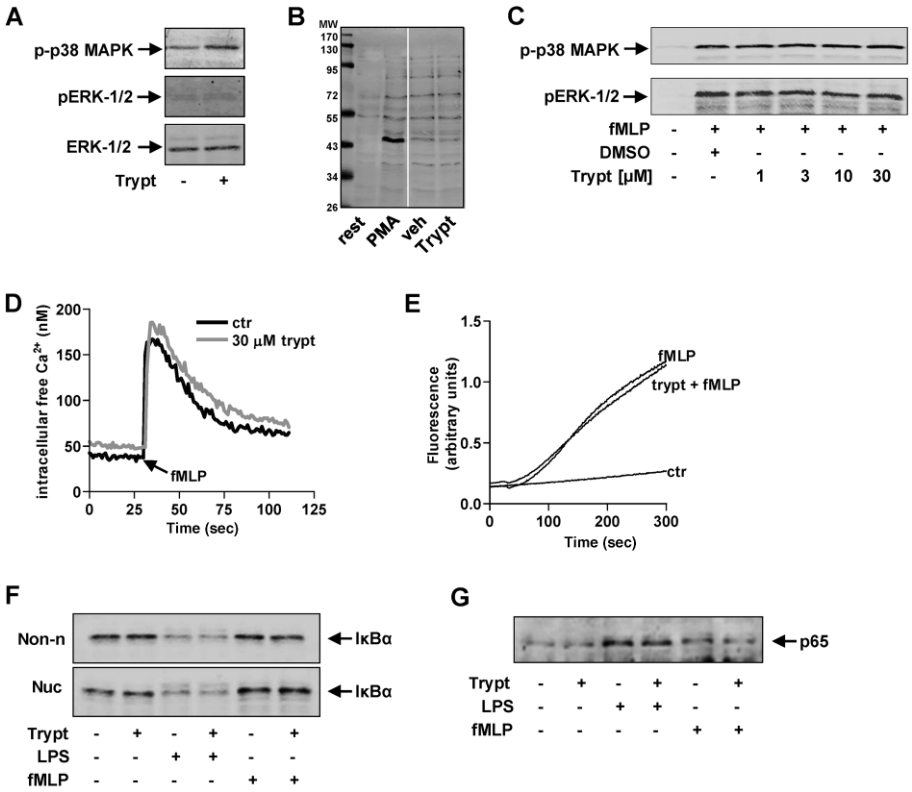


Figure 5

Effects of tryptanthrin on MAPK, $[Ca^{+2}]_i$, ROS and NF-κB activation in neutrophils. Effect of vehicle (0.1% DMSO) or tryptanthrin (30 μM, 1.5 min, 37°C) on the phosphorylation state of (A) p38 MAPK (p-p38 MAPK) and ERK1/2 (pERK1/2) and (B) of proteins containing the PKA-phosphorylation motif (RRXS/T, a marker for PKA activation). In (B), phorbol myristate acetate (PMA; 0.1 μM) was used as control. Effect of tryptanthrin on fMLP-induced increase in (C) phosphorylated ERK1/2 (pERK1/2) and p38 MAPK (p-p38 MAPK), (D) intracellular Ca^{+2} concentration, and (E) ROS formation in human neutrophils. Effect of 30 μM tryptanthrin on (F) the expression of IκBα in the non-nuclear (Non-n) and nuclear (Nuc) fractions of human neutrophils and (G) on nuclear accumulation of p65 after treatment with 1 $\mu g \cdot mL^{-1}$ LPS or 1 μM fMLP for 30 min at 37°C. The results shown are representative of at least three independent experiments.

thesis and anti-inflammatory effectiveness. The ability of a certain compound to suppress 5-LO product synthesis in whole blood is thus considered to reflect its efficacy *in vivo* (Siemoneit *et al.*, 2009). Human whole blood was pre-incubated with tryptanthrin for 10 min before stimulation with either LPS plus fMLP or with ionophore A23187. As shown in Figure 6A, tryptanthrin concentration-dependently reduced 5-LO product synthesis in whole blood with an IC_{50} of about 10 μM for both A23187 and LPS plus fMLP stimulations. Similar to neutrophils, no differences were observed in the potency of tryptanthrin for inhibition of LTB_4 and of 5-H(P)ETE in whole blood (not shown). For the 5-LO inhibitor zileuton, the IC_{50} values were 0.9 ± 0.1 μM and 2.5 ± 0.5 μM, for LPS/fMLP- and A23187-stimulated blood, respectively (Figure 6B).

Tryptanthrin inhibits LTB_4 formation in vivo

We evaluated the biological relevance of 5-LO inhibition by tryptanthrin *in vivo* in a rat model of carrageenan-induced pleurisy. The oral administration of 10 $mg \cdot kg^{-1}$ tryptanthrin to rats 1 h before carrageenan significantly reduced LTB_4 pleural levels by 46% (Figure 7A). A significant parallel inhibition of the inflammatory reaction was observed, and tryptanthrin reduced PGE_2 levels (by 42%, Figure 7B), exudate volume (by 80%, Figure 7C) and the number of infiltrating cells (by 41%, Figure 7D).

Discussion and conclusions

Here we investigated the interference by tryptanthrin of 5-LO product formation in both cell-based and cell-free systems, taking into account critical components that are relevant for the *in vivo* pharmacology of LT synthesis inhibitors. We found marked inhibition of LT formation by tryptanthrin in intact neutrophils, which is in agreement with previous findings (Danz *et al.*, 2002b). However, tryptanthrin was not found to be a direct 5-LO inhibitor, and also it did not inhibit classical signalling pathways and/or factors known to regulate 5-LO product formation in intact cells. These features suggest a novel molecular mechanism for its interference of 5-LO product formation different from other characterized blockers of LT synthesis such as inhibitors of $cPLA_2$, 5-LO or FLAP.

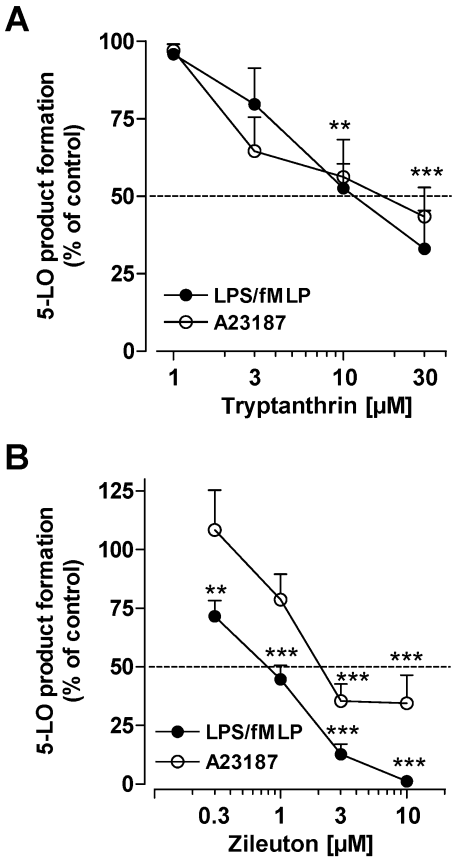


Figure 6

Tryptanthrin inhibits 5-LO product formation in human whole blood. Effect of (A) tryptanthrin and of (B) zileuton on 5-LO product synthesis in whole blood stimulated with fMLP (1 μM; 15 min, 37°C) after 30 min priming with LPS (1 μg·mL^{-1}) or with 30 μM A23187 ionophore (10 min, 37°C), as indicated. Data are expressed as percentage of control, means + SEM; $n = 3$, duplicates. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; ANOVA + Tukey's test. 5-LO products in 100% controls were (ng·mL^{-1} plasma): LPS/fMLP, 30.6 ± 10; A23187, 252.5 ± 71.9.

Importantly, tryptanthrin was active in the human whole blood assay, which is considered a suitable system for prediction of the *in vivo* pharmacodynamics of anti-LTs. Finally, oral administration of tryptanthrin was shown to be effective as an anti-inflammatory agent in the rat model of pleurisy. Our data suggest that tryptanthrin is a promising drug candidate and may represent a new approach for pharmacological intervention with LTs.

LTs are potent mediators of inflammatory and allergic reactions (Peters-Golden and Henderson, 2007), and the development of anti-LT drugs is a major challenge. However, classical concepts for inhibition of 5-LO product synthesis (e.g. interference with the catalytic redox cycle of 5-LO, chelation of the active site iron, or competition with the AA substrate) have essentially failed in drug development. Redox active compounds, and to a lesser extent also iron-ligands, are poorly selective for 5-LO, often present unfavourable pharmacokinetics, and exert severe side effects (McMillan and Walker, 1992). The potency of non-redox-type inhibitors, on the other hand, is strongly impaired by elevated peroxide levels (Werz *et al.*, 1998) and/or by phosphorylation of 5-LO by MAPKs (Fischer *et al.*, 2003), which accompany inflammatory reactions. Also, several FLAP inhibitors show poor activity in whole blood, possibly due to high plasma protein binding or competition with fatty acids (Werz, 2002). To establish new approaches, natural compounds of plant origin offer both structural diversity and privileged scaffolds. In fact, we recently identified the acylphloroglucinol hyperforin from *Hypericum perforatum* as a novel type of 5-LO inhibitor with efficacy *in vivo*, acting by interference with the C2-like domain of 5-LO (Feisst *et al.*, 2009).

Nevertheless, most of the plant-derived 5-LO inhibitors that proved effective in cell-free or cell-based systems (i.e. $IC_{50} < 50$ μM) are redox-active compounds (e.g. flavonoids, polyphenols, coumarins, quinones) and thus not immediately suitable for drug development (Werz, 2007). Other promising natural products (e.g. boswellic acids from *Boswellia* species), instead, markedly lost efficacy in whole blood and were not effective *in vivo* in human subjects (Siemoneit *et al.*, 2009). In comparison with those

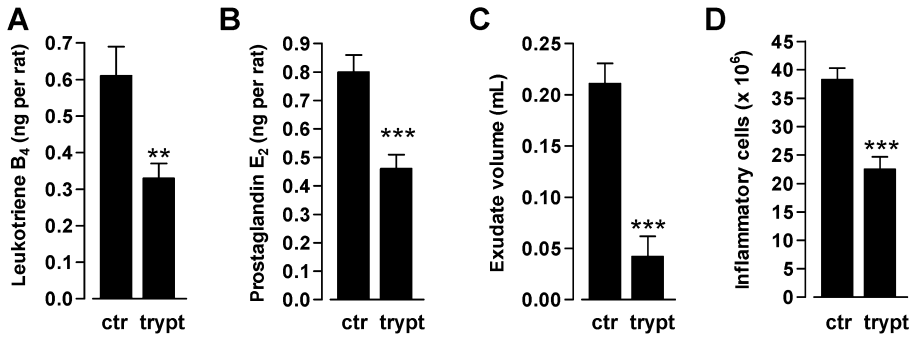


Figure 7

Tryptanthrin reduces LTB_4 levels in pleural exudates from carrageenan-treated rats. Rats ($n = 10$ for each experimental group) were treated p.o. with 10 mg·kg^{-1} tryptanthrin (trypt) or vehicle (0.5 mL of 0.5% carboxymethylcellulose and 10% Tween 20, ctr) 1 h before intrapleural injection of carrageenan. (A) LTB_4 and (B) PGE_2 pleural exudate levels, (C) exudate volume, and (D) inflammatory cell accumulation in pleural cavity were assessed 4 h after carrageenan injection. Data are expressed as mean + SEM, $n = 10$. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; Student's t test.

compounds, tryptanthrin possesses a unique, weakly basic indoloquinazoline scaffold, and our data reveal this substance as one of the most potent natural inhibitors of LT biosynthesis with proven effectiveness in whole blood and *in vivo* after oral administration. Besides tryptanthrin, the structurally related isaindigotone but also other alkaloids including colchicine, sanguinarine and chelerythrine, goshuyuamide-II, and certain alkyl-/alkylene-quinolone alkaloids have been reported to repress 5-LO product formation in intact cells (Werz, 2007). However, of these inhibitors, only isaindigotone has been found to have a direct effect on 5-LO (Molina *et al.*, 2001), whereas for all the other alkaloids inhibition of 5-LO in cell-free assays was not addressed and their molecular mechanism of action is unknown.

So far, a definite assignment of tryptanthrin to a typical class of LT synthesis inhibitors is yet not possible, but our results imply that tryptanthrin is not a direct inhibitor of 5-LO. In fact, tryptanthrin does not possess redox features and does not inhibit 5-LO in cell-free assays, independently of the peroxide tone [as for non-redox type 5-LO inhibitors (Werz *et al.*, 1998)] and of the interference with endogenous (phospho-)lipids [as for hyperforin (Feisst *et al.*, 2009)]. In addition to 5-LO, $cPLA_2$ and FLAP are also seemingly not target candidates of tryptanthrin. Thus, tryptanthrin does not inhibit AA release from $cPLA_2$ and shows different features in comparison with the FLAP inhibitor MK-886 (Kargman *et al.*, 1991; Mancini *et al.*, 1993; Fischer *et al.*, 2007). In fact, tryptanthrin only partially altered the localization of 5-LO at the nuclear membrane in stimulated cells, whereas MK886 (and also hyperforin) prevents this localization of 5-LO at the nuclear membrane. Intriguingly, however, tryptanthrin caused 5-LO redistribution, that is, the accumulation of 5-LO within the perinuclear region, as determined by immunofluorescence microscopy. After tryptanthrin treatment, 5-LO was mainly localized in the membranous area surrounding the nucleus (which is compatible with an endoplasmic reticulum staining) and only partially at the nuclear membrane, as instead observed after LPS/fMLP stimulation. We speculate that such subcellular redistribution of 5-LO may prevent activation of the enzyme and/or reduce the accessibility of 5-LO to stimuli and eventually to its substrate AA provided by FLAP. Interestingly, it has been previously observed that the 5-LO inhibitor HZ52 (Greiner *et al.*, 2011) and dual 5-LO/FLAP inhibitors of the thiopyrano[2,3,4-c,d]indole class (Hutchinson *et al.*, 1995) also cause redistribution of 5-LO from the cytosol towards the perinuclear/membrane region in the absence of stimulation. It is noteworthy that tryptanthrin potently reduced 5-LO product formation in cells stimulated with pathophysiologically-relevant stimuli (i.e. LPS and fMLP), but classical pathways activated by fMLP were not inhibited, indicating a rather specific effect on 5-LO physiology and not a general alteration of neutrophil responses.

In addition to inhibition of 5-LO product synthesis, tryptanthrin has been shown to possess other biological effects that might be beneficial in therapeutic use. On the one hand, the activity of tryptanthrin against a wide spectrum of pathogenic organisms has long been recognized (Schindler and Zahner, 1971), and its therapeutic potential as an antibacterial drug still attracts attention (Bandekar *et al.*, 2010). Sepsis belongs to those diseases that have possible associations with LTs (Peters-Golden and Henderson, 2007), and the effects of tryptanthrin on 5-LO product formation might indeed be beneficial, in particular in the second stage of sepsis, when LTs might have vasculopathic effects (Benjamim *et al.*, 2005). We showed that tryptanthrin is effective on LT synthesis when the bacterial products LPS and fMLP were used as stimuli, which prompts the evaluation of its pharmacological potential in *in vivo* experimental models of sepsis. Nevertheless, tryptanthrin might also have inhibitory effects on other relevant inflammatory pathways. In fact, we observed a reduction in the levels of both LTB_4 and PGE_2 after tryptanthrin administration in our model of rat pleurisy, which was accompanied by a reduction in exudate volume and infiltrating cells. In a previous study, we observed a similar inhibition of LTB_4 and inflammatory cells by the 5-LO inhibitors zileuton and hyperforin. On the other hand, the COX inhibitor indomethacin failed to suppress LTB_4 levels despite significant inhibition of infiltrating cells (Feisst *et al.*, 2009), which suggests that reduced LTB_4 formation in this pleurisy model is not secondary to a reduction in inflammatory cells. In accordance with the reduction in PGE_2 in the rat model of pleurisy, tryptanthrin was shown to inhibit the activity of isolated COX-2 (IC_{50} = 0.83 μM) (Danz *et al.*, 2002b) and the LPS-stimulated synthesis of PGs in a variety of cells (IC_{50} = 0.01–0.25 μM, depending on the cell type) (Danz *et al.*, 2002b). Also, the formation of NO (IC_{50} ~ 7 μM) and the expression of inducible nitric oxide synthase was impaired by tryptanthrin in murine macrophages (Ishihara *et al.*, 2000) and tryptanthrin was shown to have reduce oedema *in vivo*, in the second phase of carrageenan-induced paw oedema in mice (dosage: 50 $mg \cdot kg^{-1}$ p.o.) (Recio *et al.*, 2006), and to have beneficial effects in a murine model of inflammatory bowel disease (dosage: 100 $mg \cdot kg^{-1}$ p.o. for 3 days) (Micallef *et al.*, 2002). Whether or not tryptanthrin attenuates the activity of inflammation-related enzymes other than 5-LO by altering their subcellular location is not yet known. Interestingly, dual or multiple-target inhibitors interfering with both the LT and the PG synthetic pathways represent a major pharmacological strategy to establish a more effective anti-inflammatory therapy, with reduced number and severity of side effects (e.g. higher gastrointestinal tolerance). Tryptanthrin exhibits comparable potencies for inhibition of both LT and PG biosynthesis, which suggest a possible concomitant interference with both pathways of AA metabolism.

Recent studies have shown that tryptanthrin has antitumoural effects in leukaemia cells (Chan *et al.*, 2009) and in a breast cancer cell line MCF-7 (Yu *et al.*, 2007; 2009), indicating its potential as an anticancer agent. Although the role of 5-LO in tumour cell viability still requires further elucidation (Fischer *et al.*, 2010), inhibition of LT formation in neutrophils by tryptanthrin may reduce the inflammatory component that accompanies and sustains the development of neoplasm. Finally, tryptanthrin has two major advantages for now drugs, stability and ease of synthesis (Bandekar *et al.*, 2010). In fact, the potential of natural products in drug development has often been impaired by difficulties in isolation and/or synthesis in sufficient quantities. The synthetic accessibility of tryptanthrin also encourages research into future structural modifications for optimization of its pharmacological properties.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft. C.P. received a Carl-Zeiss stipend.

Conflicts of interest

None.

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