RESEARCH PAPER

2-(4-(Biphenyl-4-ylamino)-6-chloropyrimidin-2-ylthio)octanoic acid (HZ52) – a novel type of 5-lipoxygenase inhibitor with favourable molecular pharmacology and efficacy *in vivo*

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BACKGROUND AND PURPOSE

5-Lipoxygenase (5-LO) is the key enzyme in the biosynthesis of pro-inflammatory leukotrienes (LTs) representing a potential target for pharmacological intervention with inflammation and allergic disorders. Although many LT synthesis inhibitors are effective in simple *in vitro* test systems, they frequently fail *in vivo* due to lack of efficacy. Here, we attempted to assess the pharmacological potential of the previously identified 5-LO inhibitor 2-(4-(biphenyl-4-ylamino)-6-chloropyrimidin-2-ylthio)octanoic acid (HZ52).

EXPERIMENTAL APPROACH

We evaluated the efficacy of HZ52 *in vivo* using carrageenan-induced pleurisy in rats and platelet-activating factor (PAF)-induced lethal shock in mice. We also characterized 5-LO inhibition by HZ52 at the cellular and molecular level in comparison with other types of 5-LO inhibitor, that is, BWA4C, ZM230487 and hyperforin.

KEY RESULTS

HZ52, 1.5 mg·kg^{-1} i.p., prevented carrageenan-induced pleurisy accompanied by reduced LTB_4 levels and protected mice (10 mg·kg^{-1}, i.p.) against PAF-induced shock. Detailed analysis in cell-based and cell-free assays revealed that inhibition of 5-LO by HZ52 (i) does not depend on radical scavenging properties and is reversible; (ii) is not impaired by an increased peroxide tone or by elevated substrate concentrations; and (iii) is little affected by the cell stimulus or by phospholipids, glycerides, membranes or Ca^{2+}.

CONCLUSIONS AND IMPLICATIONS

HZ52 is a promising new type of 5-LO inhibitor with efficacy *in vivo* and with a favourable pharmacological profile. It possesses a unique 5-LO inhibitory mechanism different from classical 5-LO inhibitors and seemingly lacks the typical disadvantages of former classes of LT synthesis blockers.

Abbreviations

AA, arachidonic acid; Ada, adenosine deaminase; $cPLA_2$, cytosolic PLA_2; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DTT, dithiothreitol; EIA, enzyme immunoassay; FLAP, 5-LO-activating protein; fMLP, N-formyl-methionyl-leucyl-phenylalanine; LO, lipoxygenase; LOOH, lipid hydroperoxide; LT, leukotriene; OAG, 1-oleoyl-2-acetyl-sn-glycerol; PC, phosphatidylcholine; PMNL, polymorphonuclear leukocytes; PS, phosphatidylserine

Introduction

Leukotrienes (LTs) are local-acting messengers involved in the regulation of the immune system and are associated with inflammatory, allergic and cardiovascular disorders (Peters-Golden and Henderson, 2007). There is also increasing evidence for a pivotal role of LTs in various types of cancer (Wang and Dubois, 2010). Accordingly, these LT-related diseases might be susceptible to anti-LT agents, and LT receptor antagonists are used for treatment of asthma and allergic rhinitis (Peters-Golden and Henderson, 2007; Back, 2009). The first two steps in LT biosynthesis from arachidonic acid (AA) are catalysed by the nonhaeme iron-containing 5-lipoxygenase (5-LO), which is expressed mainly in leucocytes (Radmark *et al.*, 2007). The AA that is liberated from phospholipids in the nuclear membrane by cytosolic PLA_2 ($cPLA_2$) is converted by 5-LO into LTA_4 with the help of 5-LO-activating protein (FLAP). Further metabolism into LTB_4 or the cysteinyl LTs C_4, D_4 and E_4 occurs via additional enzymes. 5-LO is composed of a catalytic domain and a C2-like domain at the N-terminus; the latter domain is involved in binding of several regulatory factors, namely Ca^{2+}, phosphatidylcholine (PC) vesicles, 1-oleoyl-2-acetyl-sn-glycerol (OAG) and coactosine-like protein (Radmark *et al.*, 2007) and these components can compromise 5-LO inhibition (Feisst *et al.*, 2009). Activation of 5-LO is mediated by Ca^{2+} and/or phosphorylation by members of the MAPK family, and 5-LO product formation requires the presence of lipid hydroperoxides (LOOH) that oxidize the active site iron to the ferric form (Fe^{3+}) (Radmark *et al.*, 2007).

Different strategies to inhibit excessive LT formation have been pursued so far [for review, see Pergola and Werz (2010)]. Besides inhibition of $cPLA_2$ and FLAP, strong efforts have been made to find direct 5-LO inhibitors and four classes of compounds can be categorized: (i) redox-active compounds that uncouple the catalytic cycle of 5-LO by reducing the active site iron; (ii) iron ligand inhibitors such as BWA4C or zileuton; (iii) nonredox-type inhibitors that compete with AA or LOOH for binding to the enzyme (e.g. ZM230487); and (iv) structurally diverse 5-LO inhibitors, supposed to act in an allosteric manner such as hyperforin that interferes with the C2-like domain (Feisst *et al.*, 2009). Although a number of those compounds were promising drug candidates in preclinical studies, most of them were not effective in patients or failed in clinical studies due to unwanted side effects (Werz and Steinhilber, 2005). Currently, zileuton is the only 5-LO inhibitor available on the market. However, the pathophysiological actions of LTs in prevalent diseases like asthma, cardiovascular diseases and cancer urge the identification of new concepts to interfere with 5-LO and LT formation.

We have previously presented a series of α-substituted derivatives of pirinixic acid as novel and potent inhibitors of 5-LO (Koeberle *et al.*, 2008; Werz *et al.*, 2008). Previous structure-activity relationship (SAR) studies showed that introduction of a biphenyl moiety at position 6 of the pyrimidine ring improved the 5-LO inhibitory potency leading to consistent IC_{50} values between 0.4 and 2 μM in cell-free and cell-based models (Koeberle *et al.*, 2008). Here, we present the evaluation of the lead 2-(4-(biphenyl-4-ylamino)-6-chloropyrimidin-2-ylthio)octanoic acid (HZ52; Figure 1A) *in vivo* and we provide a detailed pharmacological characterization of HZ52 as a 5-LO inhibitor using cell-based and cell-free assays.

Methods

Materials

HZ52 and its corresponding ethyl carboxylate HZ49 were synthesized as described previously (Koeberle *et al.*, 2008). BWA4C and ZM230487 and BWA4C were generous gifts from Dr R. M. McMillan (Zeneca Pharmaceuticals, Macclesfield, UK) and by Dr L. G. Garland (Wellcome Res. Laboratories), respectively. AA, Ca^{2+} ionophore A23187, dithiothreitol (DTT), N-formyl-methionyl-leucyl-phenylalanine (fMLP), LPS, 1-oleoyl-2-acetyl-sn-glycerol (OAG), PC and phosphatidylserine (PS) and all other fine chemicals were from Sigma (Deisenhofen, Germany), unless stated otherwise. 13-HpODE and LTB_4 enzyme immunoassay (EIA) kit were from Cayman Chemicals (Ann Arbor, MI, USA); adenosine deaminase (Ada) and HPLC solvents were from Merck (Darmstadt, Germany). λ-Carrageenan type IV isolated from *Gigartina aciculaire* and *Gigartina pistillata* was purchased from Sigma-Aldrich (Milan, Italy). Zileuton was purchased from Sequoia Research Products (Oxford, UK).

Animals

Male adult Wistar Han rats (200–220 g; Harlan, Milan, Italy) and adult CD-1 mice (25–30 g; Harlan, Milan, Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care 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).

Carrageenan-induced pleurisy in rats

HZ52 (1.5 $mg \cdot kg^{-1}$) or zileuton (10 $mg \cdot kg^{-1}$) were given i.p. 30 min before carrageenan. Another group of rats received the vehicle (DMSO, 4%, i.p.) 30 min 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

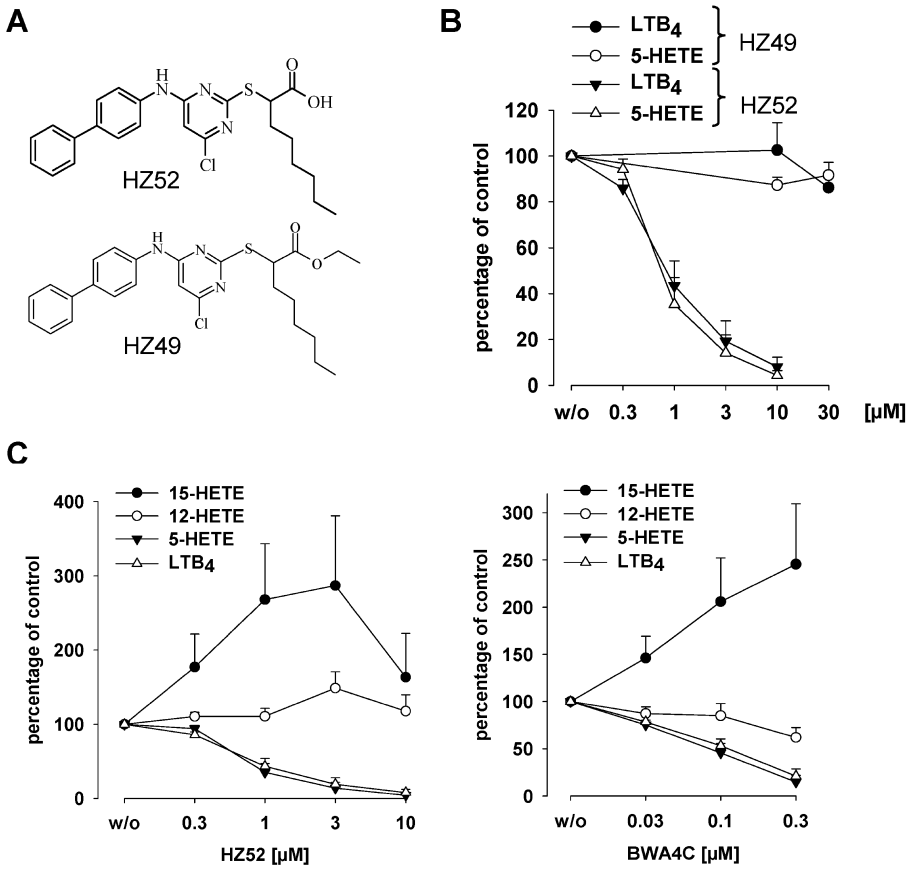


Figure 1

Inhibition of LO product synthesis in intact cells. (A) Chemical structures of HZ52 and HZ49. (B) PMNLs (5×10^6 in 1 mL of PGC buffer) were pre-incubated with HZ52, HZ49 or vehicle (0.3% DMSO, w/o) at the indicated concentrations for 15 min at 37°C. Then, 20 μM AA plus 2.5 μM A23187 was added and the reaction was stopped after 10 min. The amounts of LTB_4 and 5-H(P)ETE were determined by HPLC. The 100% values correspond to 19.6 ± 1.4 ng per 10^6 cells for LTB_4 and 181.0 ± 52.5 ng per 10^6 cells for 5-H(p)ETE. (C) PMNLs (5×10^6 in 1 mL of PGC buffer) were pre-incubated with HZ52 (left panel) or BWA4C (right panel) or vehicle (0.3% DMSO, w/o), respectively, for 15 min at 37°C. Then, 20 μM AA plus 2.5 μM A23187 was added and the reaction was stopped after 10 min. The amounts of 12-H(P)ETE, 15-H(P)ETE, LTB_4 and 5-H(P)ETE were determined by HPLC. The 100% values correspond to 17.0 ± 0.9 ng per 10^6 cells for LTB_4, 123.7 ± 11.1 ng per 10^6 cells for 5-H(p)ETE, 30.0 ± 7.3 ng per 10^6 cells for 12-H(p)ETE, 10.6 ± 2.6 ng per 10^6 cells for 15-H(p)ETE. Data are given as mean + SEM; $n = 4–5$.

dissected, and saline (0.2 mL) or λ-carrageenan type IV 1% (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 of saline solution containing heparin (5 U·mL^{-1}). The exudate and washing solution were removed by aspiration, and the total volume was measured. Any exudate that was contaminated with blood was discarded. The amount of exudates was calculated by subtracting the volume injected (2 mL) from the total volume recovered. 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 amounts of LTB_4 in the supernatant of centrifuged exudate (800× *g* for 10 min) were assayed by EIA (Cayman Chemical, Ann Arbor, MI, USA) according to manufacturer's protocol. The results are expressed as nanograms per rat and represent the mean ± SEM of 10 rats.

Platelet-activating factor-induced shock

Platelet-activating factor (PAF) C-16 was dissolved in chloroform and stored at –20°C. The PAF working solution was freshly prepared directly prior use. For this, chloroform was evaporated under N_2 and PAF was dissolved in 0.9% saline solution containing 0.25% BSA. Female mice were challenged with 200 μg·kg^{-1} PAF in a volume of 200 μL via a tail vein injection, 30 min after an i.p. injection of either vehicle (0.9% saline solution containing 2% DMSO), HZ52 or zileuton, at the indicated doses. Death (determined by cessation of breathing) was then recorded over a period of 2 h. All animals surviving the 2-h test session were killed by CO_2 inhalation.

Cell preparation

Human polymorphonuclear leucocytes (PMNL) were freshly isolated from leucocyte concentrates obtained at the Blood Center of the University Hospital Tuebingen (Germany) as described previously (Pergola *et al.*, 2008). In brief, venous blood was taken from healthy adult female donors and

leucocyte concentrates were prepared by centrifugation at 4000× *g* for 20 min at 20°C. PMNL were immediately isolated by dextran sedimentation, centrifugation on Nycoprep cushions (PAA Laboratories, Linz, Austria), and hypotonic lysis of erythrocytes. Cells were finally resuspended in PBS pH 7.4 containing 1 mg·mL^{-1} glucose and 1 mM $CaCl_2$ (PGC buffer).

Determination of product formation of 5-LO, 15-LO and 12-LO in intact cells

For assays of intact cells stimulated with Ca^{2+} ionophore A23187 or NaCl, 5 × 10^6 freshly isolated PMNL were resuspended in 1 mL of PGC buffer. After pre-incubation with the indicated test compounds for 15 min at 37°C, 5-LO product formation was started by addition of 2.5 μM A23187 and exogenous AA as indicated or 0.3 M NaCl was added 3 min before addition of AA. After 10 min at 37°C, the reaction was stopped with 1 mL of methanol and 30 μL of 1 M HCl, 200 ng PGB_1 and 500 μL of PBS were added. Formed 5-LO metabolites were extracted and analysed by HPLC as described previously (Pergola *et al.*, 2008). 5-LO product formation is expressed as nanograms of 5-LO products per 10^6 cells, which includes LTB_4 and its all-*trans* isomers, 5(S),12(S)-di-hydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid (5(S),12(S)-DiHETE) and 5(S)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-H(p)ETE). Cysteinyl LTs C_4, D_4 and E_4 were not detected, and oxidation products of LTB_4 were not determined. 15(S)-hydro(pero)xy-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid was analysed as the product of 15-LO and 12(S)-hydro(pero)xy-5,8-*cis*-10-*trans*-14-*cis*-eicosatetraenoic acid as the product of 12-LO. To assess 5-LO product formation in PMNL stimulated with fMLP, 2 × 10^7 freshly isolated PMNL were resuspended in 1 mL of PGC buffer. Cells were primed with 1 μg·mL^{-1} LPS for 10 min at 37°C and 0.3 U Ada was added. After another 10 min, cells were treated with HZ52 for 10 min and 1 μM fMLP was added to start 5-LO product formation. The reaction was stopped on ice after 5 min and cells were centrifuged (800× *g*, 10 min). The amount of LTB_4 released was determined by ELISA according to the manufacturer's protocol (Assay Designs, Ann Arbor, MI, USA).

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

Escherichia coli BL21 was transformed with pT3-5LO plasmid, and recombinant 5-LO protein was expressed at 37°C as described (Fischer *et al.*, 2003). For purification of 5-LO protein, *E. coli* were lysed by incubation in 50 mM triethanolamine HCl pH 8.0, 5 mM EDTA, soybean trypsin inhibitor (60 μg·mL^{-1}), 1 mM PMSF and lysozyme (500 μg·mL^{-1}), homogenized by sonication (3 × 15 s) and centrifuged at 40 000× *g* for 20 min. To purify 5-LO, the 40 000× *g* supernatant (S40) was applied to an ATP-agarose column (Sigma A2767; Deisenhofen, Germany). The column was eluted as described previously (Fischer *et al.*, 2003). Purified 5-LO or S40 were immediately used for 5-LO activity assays.

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

When whole cell homogenates were assayed, 5 × 10^6 freshly isolated PMNL were resuspended in 1 mL of PBS containing 1 mM EDTA (PBS/EDTA) and sonicated (3 × 10 s). Cell homogenates were either used immediately for 5-LO activity assay or centrifuged at 100 000× *g* for 1 h at 4°C and 5-LO products in the resulting 100 000× *g* supernatant (S100) were assessed. For determination of the activity of recombinant 5-LO, S40 from *E. coli* lysates (corresponding to 4 mL of *E. coli* culture) or 0.5 μg of partially purified 5-LO were diluted with PBS/EDTA. Then, 1 mM ATP was added and samples were pre-incubated with test compounds plus additional reagents (e.g. DTT, OAG or PLs) as indicated. After 5–10 min at 4°C, samples were pre-warmed for 30 s at 37°C, and 2 mM $CaCl_2$ plus AA (at the indicated concentrations) were added to start 5-LO product formation. When 13(S)-HpODE was included, the addition occurred directly prior to $CaCl_2$ plus AA. The 5-LO reaction was stopped after 10 min at 37°C by addition of 1 mL of ice-cold methanol and the formed metabolites were analysed by HPLC as described for intact cells.

Analysis of subcellular redistribution of 5-LO

PMNLs (3 × 10^7) in 1 mL PGC buffer were preincubated for 15 min at 37°C with test compounds, stimulated with 2.5 μM A23187 for 5 min, and chilled on ice. Alternatively, cells were incubated with compounds in presence of 0.3 U Ada for 20 min at 37°C (no addition of any further stimulus) and chilled on ice directly. Subcellular localization of 5-LO was determined either by mild detergent (0.1% Nonidet P-40) lysis (Pergola *et al.*, 2008) or by sonication of the cells and preparation of soluble (S100) and membrane (P100) fractions by centrifugation at 100 000× *g* (Pergola *et al.*, 2008), respectively. 5-LO in these fractions was analysed by SDS-PAGE and Western blotting using anti-5-LO antiserum (AK7, 1551, kindly provided by Dr Olof Rådmark, Stockholm, Sweden).

SDS-PAGE and Western blotting

Subcellular fractions of PMNL (20 μL, each) were mixed with 5 μL of glycerol plus 0.1% bromophenolblue (1:1, vol vol^{-1}). After SDS-PAGE on a 10% gel and electroblot to nitrocellulose membrane (Amersham), correct loading was confirmed by Ponceau S staining. The membranes were blocked with 5% BSA for 1 h at RT, washed and incubated with polyclonal anti-5-LO antiserum over night at 4°C. After washing, membranes were incubated with 1:2500 ECL Plex goat anti-rabbit IgG-Cy5 (Amersham) for 1 h at RT. Then after washing again, proteins were visualized by an Ettan™ DIGE imaging system (GE Healthcare).

DPPH assay

The radical scavenger activity was assessed by measuring the reduction of the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Blois, 1958) with slight modifications. Briefly, 100 μL of 1, 5, 25, 50, 100 μM HZ52 in ethanol (corresponding to 0.1, 0.5, 2.5, 5, 10 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 (Victor plate reader, PerkinElmer) 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.

Table 1

Effect of HZ52 on carrageenan-induced pleurisy in rats

Treatment	Exudate volume (mL)	Inflammatory cells ($\times 10^6$)	LTB_4 (ng per rat)
Vehicle	0.245 ± 0.04	55.7 ± 5.93	0.68 ± 0.09
HZ52 (1.5 mg·kg^{-1})	0.137 ± 0.025* (44%)	34.3 ± 2.91** (38%)	0.39 ± 0.04* (43%)
Zileuton (10 mg·kg^{-1})	0.06 ± 0.03** (76%)	32.9 ± 5.3* (41%)	0.23 ± 0.02*** (66%)

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus vehicle (Student's *t*-test). In parentheses, the percentages of inhibition by the test compound versus vehicle control are shown.

Thirty minutes before intrapleural injection of carrageenan, rats ($n = 10$ for each experimental group) were treated i.p. with 1.5 mg·kg^{-1} HZ52, 10 mg·kg^{-1} zileuton or vehicle (DMSO 4%). Exudate volume, LTB_4 and inflammatory cell accumulation in the pleural cavity were assessed 4 h after carrageenan injection. Data are expressed as mean ± SEM; $n = 10$.

Statistics

Data are expressed as mean ± SEM. IC_{50} values were graphically calculated from averaged measurements at 4–5 different concentrations of the compounds using SigmaPlot 9.0 (Systat Software Inc., San Jose, CA, USA). Statistical evaluation of the data was performed by one-way ANOVA followed by a Bonferroni or Tukey–Kramer *post hoc* test for multiple comparisons respectively. A *P*-value <0.05 (*) was considered significant.

Results

HZ52 exhibits anti-inflammatory efficacy in rats and protects mice against PAF-induced shock

Since many compounds that potently inhibited 5-LO *in vitro* lacked efficacy *in vivo*, we first assessed the efficacy of HZ52 in animal models related to LTs, that is, carrageenan-induced pleurisy in rats and PAF-induced shock in mice. Injection of carrageenan into the pleural cavity of rats caused an inflammatory response (within 4 h), characterized by formation of pleural exudates containing large numbers of inflammatory cells (Table 1). HZ52 (1.5 mg·kg^{-1}, i.p.) given 30 min before carrageenan significantly attenuated exudate formation (44%) and cell infiltration (38%), and reduced LTB_4 levels (43% inhibition). Zileuton (10 mg·kg^{-1}, i.p.), a clinically used 5-LO inhibitor, served as control and similarly reduced exudate formation (76%), cell infiltration (41%) and LTB_4 levels (66%) in the exudates (Table 1).

Previous studies have established a critical role for 5-LO products in the acute lethal toxicity of PAF in mice (Chen *et al.*, 1994; Goulet *et al.*, 1994; Byrum *et al.*, 1997), which thus represents a suitable model to test the *in vivo* efficacy of LT-synthesis inhibitors (Lorrain *et al.*, 2010). Administration of 200 μg·kg^{-1} PAF to vehicle-treated mice caused 95% mortality within 30 min, hence only one out of 20 animals survived. HZ52 at the dose of 10 mg·kg^{-1}, but not at 1.5 or 5 mg·kg^{-1} (i.p.), led to survival of eight out of 10 animals (Table 2). Treatment with the 5-LO inhibitor zileuton, at the dose of 10 mg·kg^{-1} (i.p.), prior to the PAF injection resulted in 7 out of 11 mice surviving the PAF treatment. Taken together, these results indicate that HZ52 is about equally effective as the 5-LO inhibitor zileuton in the LT-related models of carrageenan-induced pleurisy and PAF-induced shock.

Table 2

HZ52 protects mice against PAF-induced shock

Compound	Survivors per total
Vehicle	1/20
HZ52 1.5 mg·kg^{-1}	0/5
HZ52 5 mg·kg^{-1}	0/5
HZ52 10 mg·kg^{-1}	8/10
Zileuton 10 mg·kg^{-1}	7/11

Platelet-activating factor was injected i.v. into female mice at a dose of 200 μg·kg^{-1}. HZ52, zileuton or vehicle control (saline solution containing 2% DMSO) was administered i.p. 30 min prior to PAF. The number of surviving animals and the total number of animals tested for each group are given.

Inhibition of 5-LO product formation by HZ52 in cell-based models

The effectiveness of HZ52 observed *in vivo* motivated us to explore its cellular and molecular pharmacology in more detail. In accordance with our previous findings (Koeberle *et al.*, 2008), HZ52 inhibited 5-LO product synthesis in human PMNL stimulated with the Ca^{2+} ionophore A23187 (2.5 μM) plus AA (20 μM) with an $IC_{50} = 0.7$ μM for both LTB_4 and 5-H(P)ETE (Figure 1B). HZ49, the corresponding ethyl ester of HZ52, was not active up to 30 μM (Figure 1B), indicating that the free carboxylic group is essential for bioactivity. To investigate the selectivity of HZ52 for 5-LO, we monitored the formation of 12-H(P)ETE and 15-H(P)ETE in the PMNL incubations resulting from platelet-type 12-LO (present in PMNL-adherent platelets) and from 12/15-LO (15-LO-1), expressed in eosinophilic granulocytes. The amount of 12-H(P)ETE was not decreased in PMNL stimulated with A23187 plus 20 μM AA but the formation of 15-H(P)ETE was rather augmented (Figure 1C). A similar profile could be observed for BWA4C (Tateson *et al.*, 1988) used as reference 5-LO inhibitor.

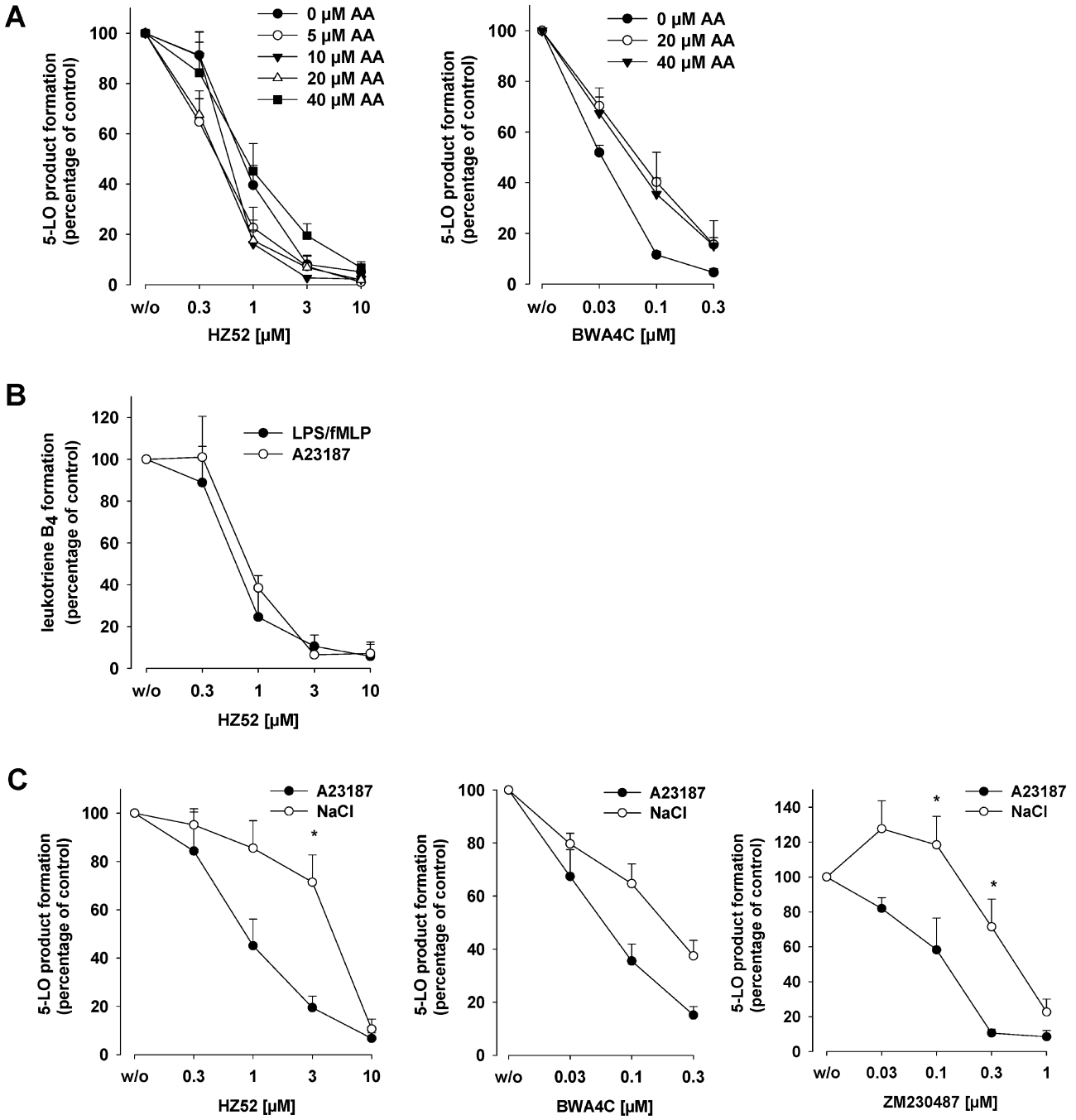


Figure 2

Effects of HZ52 on 5-LO product formation in PMNL induced by different stimuli. (A) PMNLs (5×10^6 in 1 mL of PGC buffer) were pre-incubated with HZ52, BWA4C or vehicle (0.3% DMSO, w/o) for 15 min at 37°C and then stimulated with 2.5 µM A23187 plus the indicated amounts of AA. After 10 min at 37°C, the reaction was stopped and 5-LO products were assessed. The 100% values correspond to 39.8 ± 7.6, 65.7 ± 10.7, 106.1 ± 38.1, 227.3 ± 59.9 and 105.9 ± 12.2 ng per 10^6 cells of 5-LO products at 0, 5, 10, 20 and 40 µM AA, respectively. Values are given as means + SEM; $n = 4$. (B) PMNLs (5×10^6 in 1 mL of PGC buffer) were pre-incubated with HZ52 for 15 min at 37°C. Then, 2.5 µM A23187 was added and after 10 min LTB_4 was determined by HPLC. The 100% values correspond to 17.3 ± 8.7 ng per 10^6 cells for LTB_4. Alternatively, PMNLs (2×10^7) were primed with 1 $\mu g \cdot mL^{-1}$ LPS for 10 min at 37°C and 0.3 U Ada was added. After another 10 min, cells were treated with HZ52 for 10 min and 1 µM fMLP was added. The reaction was stopped on ice after 5 min and released LTB_4 was determined by ELISA. The 100% values correspond to 9.5 ± 2.9 pg per 10^6 cells for LTB_4. Values are given as means + SEM; $n = 3$. (C) PMNL (5×10^6 in 1 mL of PGC buffer) were pre-incubated with HZ52, BWA4C or ZM230487 for 15 min at 37°C. NaCl (0.3 M) was added 3 min before addition of 40 µM AA or cells were stimulated with 2.5 µM A23187 in the presence of 40 µM AA. After 10 min at 37°C, the reaction was stopped and 5-LO products were assessed. The 100% values correspond to 105.9 ± 12.2 and 67.2 ± 10.0 ng per 10^6 cells for 5-LO products for cells stimulated by A23187 and NaCl, respectively. Values are given as means + SEM; $n = 4$; $^*P < 0.05$ versus controls that were stimulated with A23187, ANOVA + Tukey *post hoc* test.

Suppression of cellular 5-LO product formation by an inhibitor may occur by competition with the substrate, in particular by so-called competitive 5-LO inhibitors or FLAP antagonists (Werz *et al.*, 1998; Fischer *et al.*, 2007). After supplementation of PMNL with different amounts of exogenous substrate (up to 40 μM AA), however, only slight, non-significant differences in the potency of HZ52 were observed, and 10 μM HZ52 always produced a total suppression of 5-LO product formation (Figure 2A). For BWA4C, exogenous AA also slightly reduced the potency (Figure 2A), whereas for the FLAP antagonists, licofelone and MK886, a strong loss of efficacy was evident (Fischer *et al.*, 2007).

Previous studies showed that the efficacy of 5-LO inhibitors might depend on the stimulus (Werz *et al.*, 1998; Fischer *et al.*, 2003). Elicitation of 5-LO product formation in PMNL by the Ca^{2+} ionophore A23187 is due to a robust Ca^{2+} influx into the cells. In contrast, the bacterial peptide fMLP (a pathophysiologically relevant stimulus) activates cellular 5-LO via GPCR signalling due to elevated $[Ca^{2+}]_i$ and phosphorylation events (Werz *et al.*, 2002). HZ52 suppressed LTB_4 formation in PMNL stimulated with fMLP [1 μM, upon priming with 1 $\mu g \cdot mL^{-1}$ LPS and 0.3 $U \cdot mL^{-1}$ Ada (Pergola *et al.*, 2008)] with the same potency and efficacy as with A23187 stimulation (Figure 2B). Another means to elicit 5-LO product formation in PMNL is the induction of hyperosmotic cell stress that activates p38 MAPK-dependent MAPKAPK-2 which phosphorylates and thus activates 5-LO without the need for an elevated $[Ca^{2+}]_i$. HZ52 (10 μM) neither induced activation of ERK or p38 MAPK nor prevented the fMLP-evoked activation of these kinases (data not shown). On the other hand, such phosphorylations attenuate the efficacy of nonredox-type 5-LO inhibitors [e.g. ZM230487 (Fischer *et al.*, 2003)], and in fact when PMNL were exposed to 300 mM NaCl plus 40 μM AA the efficacy of ZM230487 (control) was clearly reduced whereas the potency of BWA4C was less affected, as expected (Werz *et al.*, 1998; Figure 2C). Also for HZ52, the potency was decreased when PMNL were challenged by hypertonicity (Figure 2C).

HZ52 promotes 5-LO translocation to the nuclear membrane in PMNL

After stimulation of neutrophils, 5-LO translocates from the cytosol to the perinuclear region and becomes membrane-associated (Radmark *et al.*, 2007). It is assumed that the mobile portion of 5-LO translocating to the nucleus is responsible for LT synthesis. In fact, agents such as FLAP inhibitors (Brideau *et al.*, 1992), hyperforin (Feisst *et al.*, 2009) or sulindac sulphide (Steinbrink *et al.*, 2010) were shown to block 5-LO translocation to the nucleus and thus, can effectively repress 5-LO product synthesis, although inhibition of 5-LO translocation by FLAP inhibitors might be an artefact. We investigated the effects of HZ52 on the subcellular localization of 5-LO by means of a crude cell fractionation using mild detergent lysis (0.1% NP-40; compare Pergola *et al.*, 2008). Pre-incubation of PMNL with HZ52 (3 to 30 μM) and subsequent stimulation with A23187 induced a concentration-dependent 5-LO accumulation in the nuclear fraction. In contrast, hyperforin (30 μM) prevented the accumulation of 5-LO in the nuclear fraction (Figure 3A). Interestingly, treatment of PMNL with HZ52 (10 and 30 μM) alone (in the presence or absence of Ada) caused a distinct enrichment of 5-LO at the nuclear fraction (Figure 3B). Notably, HZ49, the inactive ethyl ester of HZ52, had no effect on 5-LO distribution, suggesting the importance of the acidic group for this process. Finally, PMNL were completely disrupted by sonification after treatment with HZ52 (10 or 30 μM) and separated into a soluble and a membraneous fraction by 100 000× *g* centrifugation. Figure 3C shows that HZ52 directs 5-LO to the membrane compartment.

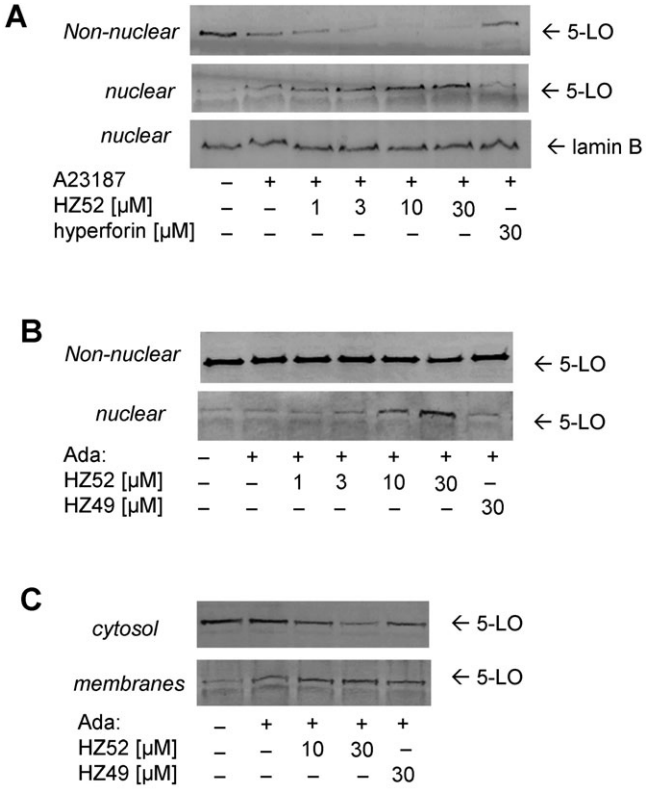


Figure 3

HZ52 induces 5-LO translocation to the perinuclear membrane region. (A, B) 5-LO was analysed in nuclear and non-nuclear fractions of PMNL (lysed by 0.1% NP-40) or (C) in the soluble (S100) and membrane (P100) fractions after 100 000× *g* centrifugation of lysates obtained by sonification of PMNL by SDS-PAGE and immunoblotting after subcellular fractionation. (A) PMNLs (3×10^7 in 1 mL of PGC buffer) were pre-incubated with HZ52 at the indicated concentrations, hyperforin (30 μM) or with vehicle (0.3% DMSO) for 15 min at 37°C. Then, 2.5 μM A23187 or vehicle (0.25% DMSO) was added and cells were incubated for another 5 min at 37°C. (B, C) PMNL were incubated with HZ52 at the indicated concentrations, hyperforin (30 μM), HZ49 (30 μM) or with vehicle (0.3% DMSO) in the presence of 0.3 U Ada for 20 min at 37°C. Results are representative of similar results obtained in two additional independent experiments.

Inhibition of 5-LO activity by HZ52 in cell-free assays

As found before (Koeberle *et al.*, 2008), HZ52 (but not the corresponding ethyl ester HZ49) also inhibits 5-LO in cell-free assays using partially purified recombinant 5-LO enzyme

with an IC_{50} = 1.5 μM, implying a direct interference of the compound with 5-LO. Since most of the direct 5-LO inhibitors act in a reversible manner, we first tested whether the reduction in 5-LO product formation by HZ52 is reversible. Partially purified 5-LO enzyme was pre-incubated with 3 μM HZ52. After 10-fold dilution with assay buffer, 5-LO product formation was almost at the same level as after treatment with 0.3 μM HZ52 without prior dilution (Figure 4A). As a control, the inhibition of the iron ligand-type 5-LO inhibitor BWA4C was reversed after 10-fold dilution as well (Figure 4A).

Typically, redox-active compounds inhibit 5-LO by reducing the active-site iron to the ferrous form thus interrupting the catalytic cycle of 5-LO. To analyse whether HZ52 possesses reducing/radical scavenging properties, the compound was tested for its ability to reduce DPPH in the presence of a hydrogen donor. HZ52 failed to reduce DPPH (Figure 4B) whereas L-cysteine and ascorbic acid (serving as positive controls) acted as expected.

To test whether the effect of HZ52 depends on the 5-LO substrate concentration, the amount of AA in the cell-free assay was varied. Interestingly, at AA concentrations ≤7.5 μM the IC_{50} values were 7 to 8 μM, whereas at AA concentrations of 10 to 40 μM the potency of HZ52 was significantly increased with IC_{50} values = 1.3 to 1.5 μM (Figure 4C). Thus, there is a considerable shift of the efficacy between 7.5 μM and 10 μM AA. In contrast, the potency of BWA4C was not affected by varying the AA concentration (Figure 4C).

Among 5-LO inhibitors, the nonredox-type representatives [e.g. ZM230487 (Werz *et al.*, 1998)] are much more potent in intact cells (at low LOOH levels) as compared to cell-free test systems where LOOH are abundant (Werz *et al.*, 1998). The ability of HZ52 to inhibit purified 5-LO (IC_{50} = 1.5 μM) was actually not markedly impaired versus intact PMNL (IC_{50} = 0.7 μM) but a reduction was observed in PMNL homogenates (IC_{50} = 9 μM) or corresponding 100 000× *g* supernatants (S100, IC_{50} = 8 μM). When 1 mM DTT was given to homogenates or S100 (to reconstitute glutathione peroxidase activity and thus to lower the LOOH levels) the potency of HZ52 or of BWA4C was not altered (Figure 4D), which is in contrast to the strong shift in the potency of ZM230487 (Werz *et al.*, 1998). Also, in contrast to ZM230487, addition of 13-(S)-HpODE (1 μM) did not affect the inhibition of purified 5-LO by HZ52 (Figure 4E) or BWA4C (not shown).

Effects of 5-LO modulatory factors on the efficacy of HZ52

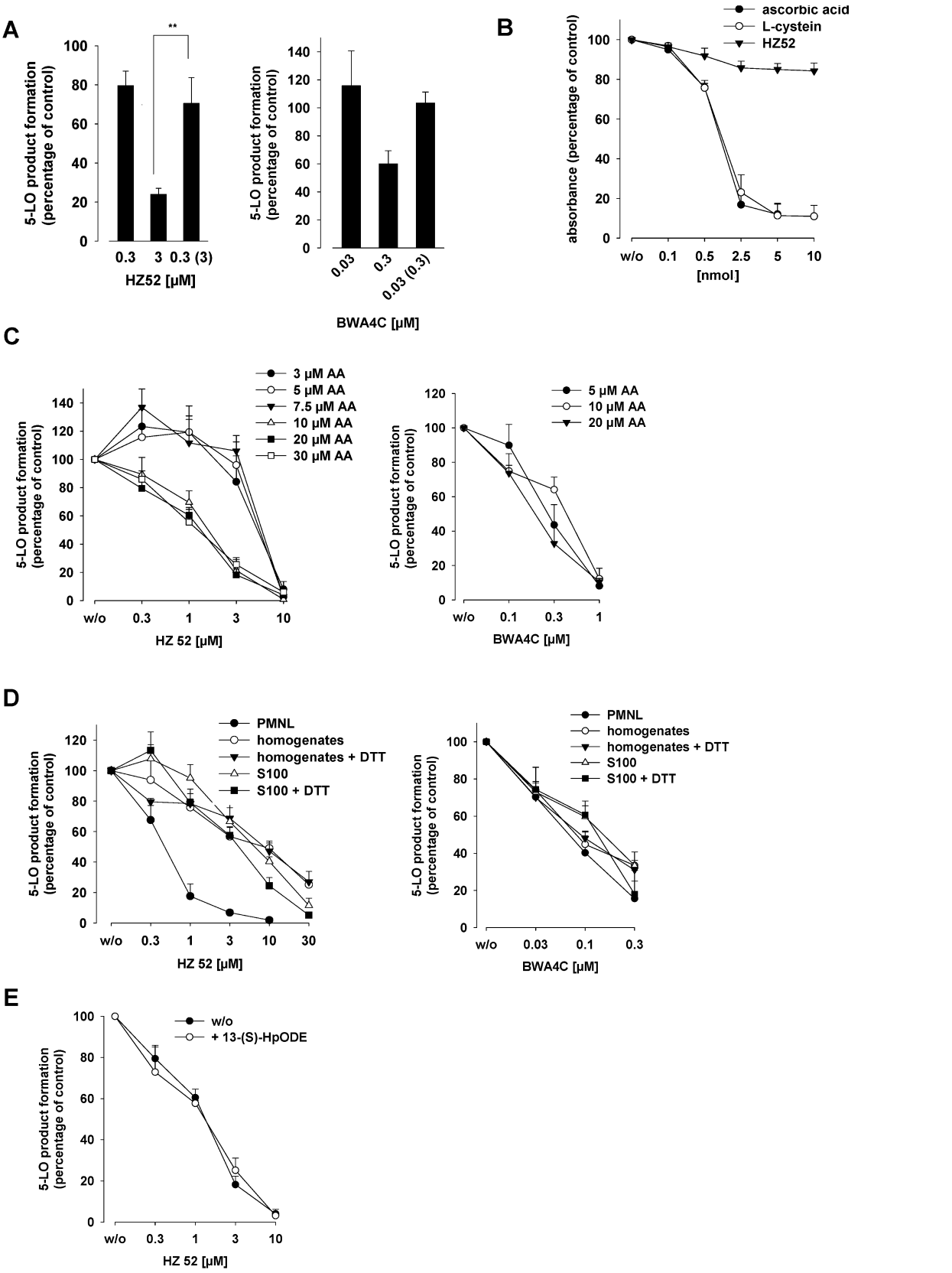
Since HZ52 lost some potency in homogenates versus isolated 5-LO, cellular components may attenuate its 5-LO inhibitory effect. Such reduced potency in homogenates has also been observed for hyperforin, and indeed, PC abolished 5-LO inhibition by hyperforin (Feisst *et al.*, 2009). Addition of PC (25 μg·mL^{-1}) to purified 5-LO slightly but significantly attenuated the potency of HZ52, whereas PS had no significant effect. The efficacy of BWA4C was marginally decreased by PC (Figure 5A). Inhibition of purified 5-LO by HZ52 in the presence or absence of Ca^{2+} was instead comparable (Figure 5B), excluding a primary interference of HZ52 with Ca^{2+} binding within the C2 domain. Finally, we investigated if OAG, which also activates 5-LO at the C2-like domain and reduces the efficacy of hyperforin (Feisst *et al.*, 2009), modulates the 5-LO inhibitory effect of HZ52. OAG (30 μM) moderately impaired the potency of HZ52 to inhibit purified 5-LO, which was not the case for BWA4C (Figure 5C).

Discussion and conclusions

The α-substituted pirinixic acid derivative HZ52 was recently identified as lead compound within a series of novel 5-LO

Figure 4

Inhibition of 5-LO activity by HZ52 in cell-free assays. (A) Partially purified human recombinant 5-LO (0.5 μg in 1 mL of PBS/EDTA/ATP) was pre-incubated with 3 μM HZ52 or 0.3 μM BWA4C for 10 min at 4°C. Then, one aliquot was diluted with assay buffer 10-fold, whereas the other one was not altered, and 20 μM AA plus 2 mM Ca^{2+} were added to start the reaction. For comparison, 5-LO (0.5 μg) was pre-incubated with 0.3 μM HZ52 or 0.03 μM BWA4C, and then 20 μM AA plus 1 mM Ca^{2+} were added (no dilution). Then, all samples were incubated for 10 min at 37°C and 5-LO product formation was analysed as described. The 100% values correspond to 609.4 ± 74.5 ng 5-LO products per 0.5 μg enzyme. Values are given as means + SEM; $n = 3$; $^{**}P < 0.01$ versus inhibition w/o dilution, ANOVA + Bonferroni *post hoc* test. (B) Radical scavenging properties. HZ52, L-cysteine or ascorbic acid (at the indicated amounts) were incubated with 5 nmol DPPH in 100 μL of EtOH for 30 min at RT and the absorbance at 520 nm was measured. Values are given as means + SEM; $n = 3$. (C) Partially purified 5-LO (0.5 μg in 1 mL of PBS/EDTA/ATP) was pre-incubated with HZ52 (left panel) or BWA4C (right panel) as indicated or with vehicle (0.3% DMSO, w/o) for 5–10 min at 4°C. Samples were pre-warmed at 37°C for 30 s and 5-LO product formation was started by addition of 2 mM $CaCl_2$ together with AA at the indicated concentrations. After 10 min, 5-LO product formation was determined. The 100% values correspond to 107.7 ± 23.7, 255.8 ± 34.1, 343.8 ± 19.4, 587.2 ± 83.1, 923.4 ± 196.11 and 499.6 ± 258.5 ng 5-LO products per 0.5 μg of enzyme at 3, 5, 7.5, 10, 20 and 30 μM AA, respectively. Values are given as mean + SEM; $n = 3$–5. (D) PMNL (5×10^6) were sonicated and the homogenates or corresponding supernatants (S100) derived from 100 000× *g* centrifugation were pre-incubated with HZ52 (left panel) or BWA4C (right panel) or vehicle (0.3% DMSO, w/o) in PBS/EDTA/ATP at 4°C for 5 min. Then, 1 mM DTT (as indicated) was added, and after another 5 min, samples were pre-warmed at 37°C for 30 s and 5-LO product formation was started by addition of 2 mM $CaCl_2$ plus 20 μM AA. After 10 min, 5-LO products were determined. Inhibition of 5-LO activity in intact PMNL is given for comparison. The 100% values correspond to 153.0 ± 52.3, 209.0 ± 52.6, 112.1 ± 13.3, 140.0 ± 22.8 and 227.3 ± 59.9 ng 5-LO products per 10^6 cells in homogenates, homogenates plus DTT, S100, S100 plus DTT and intact PMNLs, respectively. Values are given as mean + SEM; $n = 3$–4. (E) Partially purified 5-LO (0.5 μg) was pre-incubated with HZ52 or vehicle (0.3% DMSO, w/o) in PBS/ETDA/ATP as indicated for 5–10 min at 4°C. Samples were pre-warmed at 37°C for 30 s and 5-LO product formation was started by addition of 2 mM $CaCl_2$ plus 20 μM AA in presence or absence of 13(S)-HpODE (1 μM). After another 10 min, 5-LO products were assessed. The 100% values correspond to 923.4 ± 196.11 and 1373.9 ± 197.4 ng 5-LO products per 0.5 μg of enzyme in the absence and presence of 13(S)-HpODE, respectively. Values are given as mean + SEM; $n = 3$.


A
**
5-LO product formation (percentage of control)
0.3
3
0.3 (3)
HZ52 [μM]
0.03
0.3
0.03 (0.3)
BWA4C [μM]
B
ascorbic acid
L-cystein
HZ52
absorbance (percentage of control)
w/o
0.1
0.5
2.5
5
10
[nmol]
C
3 μM AA
5 μM AA
7.5 μM AA
10 μM AA
20 μM AA
30 μM AA
HZ 52 [μM]
BWA4C [μM]
D
PMNL
homogenates
homogenates + DTT
S100
S100 + DTT
HZ 52 [μM]
BWA4C [μM]
E
w/o
+ 13-(S)-HpODE
HZ 52 [μM]

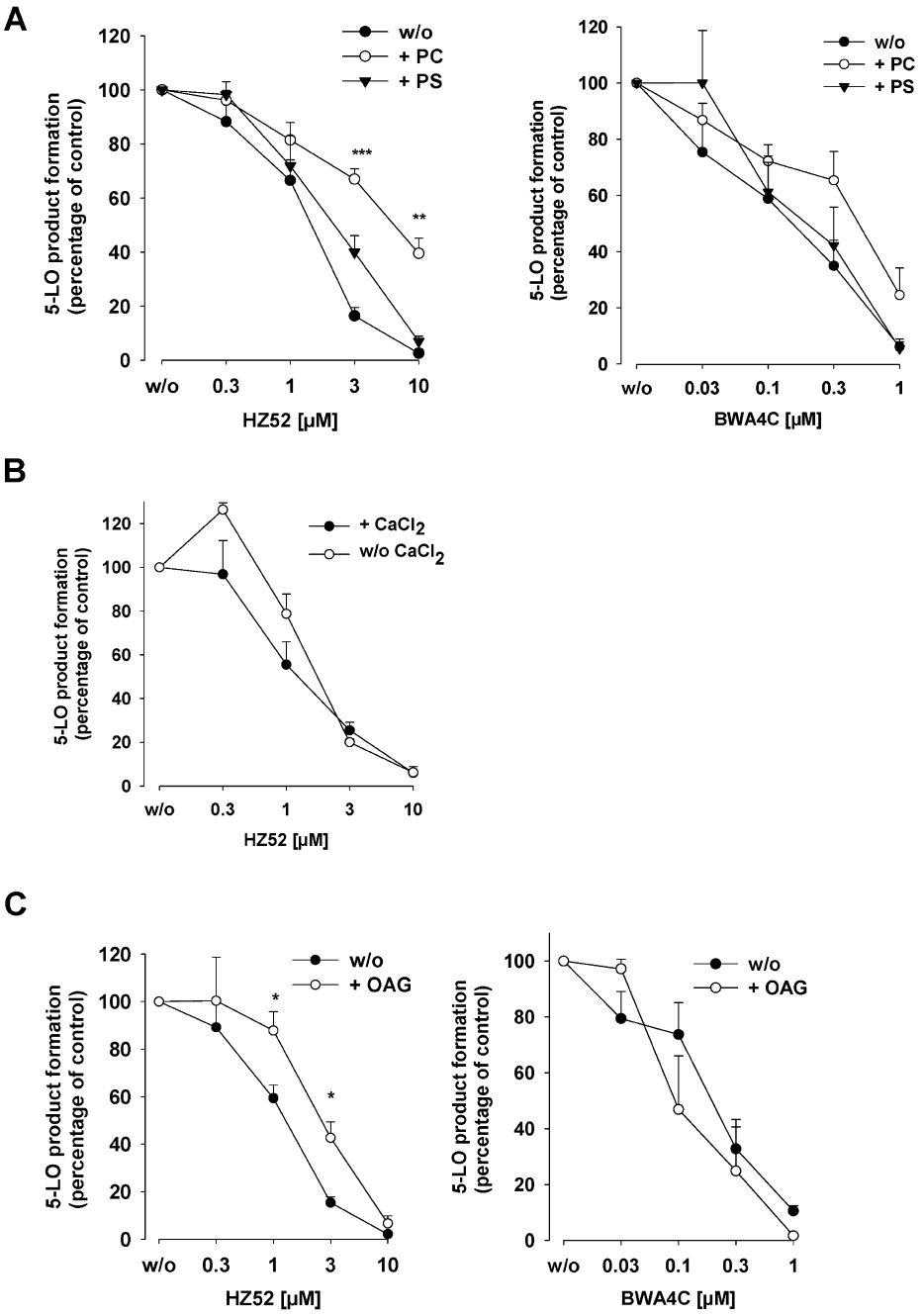


Figure 5

Cellular components and lipids attenuate the 5-LO inhibitory potency of HZ52. (A) Partially purified 5-LO (0.5 µg in 1 mL of PBS/EDTA/ATP) was pre-incubated with HZ52 (left panel) or BWA4C (right panel) or with vehicle (0.3% DMSO, w/o) for 5–10 min at 4°C with or without PC or PS (25 µg·mL^{-1}, each) as indicated. Samples were pre-warmed at 37°C for 30 s and 5-LO product formation was started by addition of 2 mM $CaCl_2$ plus 20 µM AA. After another 10 min, 5-LO products were determined. The 100% values correspond to 874.7 ± 290.0, 1375.0 ± 111.3 and 897.5 ± 96.3 ng 5-LO products per 0.5 µg of enzyme in the absence or presence of PC and PS, respectively. Values are given as mean + SEM; n = 4–5. (B) Partially purified 5-LO (0.5 µg in 1 mL of PBS/EDTA/ATP) was pre-incubated with HZ52 or vehicle (0.3% DMSO, w/o) for 5–10 min at 4°C. Samples were pre-warmed at 37°C for 30 s and 5-LO product formation was started by addition of 30 µM AA with or without 2 mM $CaCl_2$. After 10 min, 5-LO products were determined. The 100% values correspond to 1353.5 ± 405.3 and 499.6 ± 258.5 ng 5-LO products per 0.5 µg of enzyme in the absence or presence of Ca^{2+}, respectively. Values are given as mean + SEM; n = 3. (C) Partially purified 5-LO (0.5 µg in 1 mL of PBS/EDTA/ATP) was pre-incubated with HZ52 (left panel) BWA4C (right panel) or with vehicle (0.3% DMSO, w/o) for 5–10 min at 4°C with OAG (30 µM) or solvent (0.3% DMSO). Samples were pre-warmed at 37°C for 30 s and 2 mM $CaCl_2$ plus 20 µM AA was added. After another 10 min, 5-LO products were determined. The 100% values correspond to 1155.6 ± 138.3 and 801.9 ± 124.3 ng 5-LO products per 0.5 µg of enzyme in the absence or presence of OAG respectively. Data are means + SEM; $n \geq 3$; *$P < 0.05$ versus controls without OAG (w/o) at corresponding concentrations of test compounds, ANOVA + Tukey *post hoc* test.

inhibitors with distinct SARs (Koeberle *et al.*, 2008; Werz *et al.*, 2008). Here, we assessed the *in vivo* efficacy and anti-inflammatory activity of HZ52, and we characterized its pharmacological profile in order to judge the value of further development. The efficacy of HZ52 in the rat pleurisy and in the murine model of PAF-induced shock clearly proves that it has *in vivo* activity. Our biochemical analysis revealed HZ52 to be a novel type of direct 5-LO inhibitor with distinct characteristics as compared to classical redox-type, iron ligand-type, nonredox-type inhibitors or 5-LO C2-like domain inhibitors. Obviously, certain disadvantages of other types of 5-LO inhibitors that blighted their successful development were less noticeable in HZ52, thus indicating a favourable profile and encouraging pharmacodynamic features of HZ52 worthy of further development as an anti-inflammatory drug.

Numerous small molecules from natural sources and of synthetic origin have been described during the past three decades as 5-LO product synthesis inhibitors (Werz, 2007; Pergola and Werz, 2010). However, despite the huge number and strong diversity of such compounds only zileuton, a hydroxurea derivative chelating and reducing the active site iron in 5-LO (Carter *et al.*, 1991), has been approved by the FDA and reached the market. Many compounds were not further developed based on their lack of efficacy *in vivo* and low therapeutic potential for treatment of LT-related diseases (Peters-Golden and Henderson, 2007). Apparently, the distinct conditions at sites of inflammation hampered the efficacy of these substances *in vivo* (Werz and Steinhilber, 2005). Our previous studies revealed that HZ52 is a direct 5-LO inhibitor with high potency in cell-free and cell-based *in vitro* assays. Thus, HZ52 inhibited the activity of isolated 5-LO with IC_{50} = 1.5 μM and blocked 5-LO product formation in intact PMNL stimulated with A23187 in the presence of exogenous AA with an IC_{50} = 0.7 μM (Koeberle *et al.*, 2008). Here, we show that HZ52 suppresses 5-LO product synthesis *in vivo*, visualized by the reduced LTB_4 levels in the pleural exudates of carrageenan-treated rats after a low-dose treatment, 1.5 $mg \cdot kg^{-1}$, and this was accompanied by a significant reduction in the inflammatory response. In addition to the pleurisy model, which is a routine test system for evaluation of LT synthesis inhibitors (Ku *et al.*, 1988; Smith *et al.*, 1992; Raychaudhuri *et al.*, 1997; Cuzzocrea *et al.*, 2003), we used another 5-LO-related disease model, that is, PAF-induced lethal shock in mice (Chen *et al.*, 1994; Goulet *et al.*, 1994; Byrum *et al.*, 1997). This model is used as predictive *in vivo* test system for analysis of anti-LTs (Lorrain *et al.*, 2010). For both carrageenan-induced pleurisy and PAF-induced shock, studies with 5-LO-deficient mice revealed a significant functional role of 5-LO (Chen *et al.*, 1994; Cuzzocrea *et al.*, 2003). Indeed, HZ52 significantly protected the mice treated with PAF against the lethal shock, and side-by-side comparison with zileuton at the same dose (i.e. 10 $mg \cdot kg^{-1}$) shows an equal or even higher efficacy for HZ52. The equivalent efficacy *in vivo* is reflected by comparable IC_{50} values of HZ52 (0.4–1 μM) and zileuton [0.5–1 μM (Carter *et al.*, 1991)] in various cell-based 5-LO activity assays *in vitro*. Hence, our data allow us to designate HZ52 as a direct 5-LO inhibitor with considerable efficacy *in vivo*. Although the blood–brain permeability of HZ52 is not known, its lipophilic structure suggests availability in the brain, which is of interest with respect to the proposed beneficial effects of 5-LO inhibitors in Alzheimer's disease (Chu and Pratico, 2011).

Direct 5-LO inhibitors can be classified into redox-active substances, iron-ligand inhibitors, nonredox-type inhibitors and compounds with different modes of action (Pergola and Werz, 2010). Based on our data, HZ52 cannot be unequivocally grouped into one class suggesting a novel, thus far unrecognized molecular mode of action for its ability to interfere with 5-LO activity. Direct interference with ionophore can be excluded since HZ52 suppressed 5-LO product formation induced by LPS and fMLP almost equally well. Since HZ52 inhibited cellular 5-LO product synthesis in the absence or presence of 20 μM exogenous AA equally well, we suggest that suppression of release of AA is also unlikely. Redox-active 5-LO inhibitors are highly potent *in vitro* but most of them were not further developed due to rapid inactivation *in vivo* and lack of selectivity (McMillan and Walker, 1992; Hutchinson *et al.*, 1995). Thus, interference with other redox systems caused unwanted toxic side effects (McMillan and Walker, 1992). The lack of moieties with reducing properties in the overall structure of HZ52 and the failure to exhibit any radical scavenging/reducing effect in the DPPH assay obviously exclude a redox- or radical scavenging-based 5-LO inhibitory mechanism. Also, no typical iron-chelating moiety of prototype iron-ligand inhibitors [e.g. hydroxamic acid in BWA4C (Tateson *et al.*, 1988) or hydroxyurea in zileuton (Carter *et al.*, 1991)] is evident. Along these lines, HZ52 failed to inhibit the iron-containing and redox-sensitive 12- and 15-LOs.

In contrast to nonredox-type inhibitors [e.g. ZM230487, L-739.010 and CJ-13.610 (Werz *et al.*, 1998; Fischer *et al.*, 2004)], suppression of 5-LO activity by HZ52 was not influenced by elevated peroxide levels or by the redox tone. This is an advantageous property of HZ52, since oxidative stress and an altered redox tone are often associated with LT-related diseases and compromise the efficacy of nonredox-type 5-LO inhibitors [see Werz *et al.* (1998) and references therein]. In intact PMNL, HZ52 inhibited 5-LO product synthesis equally well when stimulated by LPS plus fMLP or by A23187, which is again in contrast to nonredox-type 5-LO inhibitors that are more efficient in A23187-elicited PMNL (Fischer *et al.*, 2003). On the other hand, HZ52 shares with nonredox-type 5-LO inhibitors a reduced potency in PMNL challenged by hypertonic stress.

HZ52 also showed some characteristics of the C2-like domain inhibitor hyperforin. For example, in contrast to BWA4C, the interference of HZ52 with 5-LO was partly reversed by PC (but not by PS) or OAG, and cellular components (present in homogenates) impaired the potency of HZ52 as well. However, the magnitude of modulation of HZ52-mediated 5-LO inhibition by these factors was markedly weak. Depletion of Ca^{2+} in cell-free assays did not alter the profile of HZ52. Therefore, we exclude an interference of HZ52 with the Ca^{2+}-binding residues within the C2-like domain of 5-LO. Also, HZ52 did not prevent Ca^{2+}-mediated nuclear 5-LO translocation and membrane association, another property of C2 domain-interfering agents (Kulkarni *et al.*, 2002; Feisst *et al.*, 2009). In contrast, HZ52 caused redistribution of 5-LO from the cytosol towards the perinuclear (membrane) region, and this occurred in the absence or presence of any stimulus. Similarly, thiopyrano[2,3,4-c,d]indoles,

a class of dual 5-LO/FLAP inhibitors, promoted translocation of 5-LO to the nuclear membrane also in the absence of any stimulus (Hutchinson *et al.*, 1995). Nevertheless, higher concentrations of HZ52 were needed to induce nuclear redistribution (10 to 30 μM) compared to those required to substantially inhibit 5-LO product synthesis in A23187 or fMLP/LPS-stimulated PMNL (IC_{50} = 0.4–0.7 μM), suggesting that the redistribution is probably not the primary cause for 5-LO inhibition in PMNL. Together these results suggest that HZ52 primarily acts by distinct mechanisms other than interference with the functionality of the 5-LO C2-like domain.

One of the remarkable characteristics of HZ52 is that its potency in the cell-free assay depends on the substrate concentration, in that HZ52 possessed a definite lower potency (IC_{50} = 7–8 μM) at AA concentrations <7.5 μM and high potency (IC_{50} = 1.3–1.5 μM) at AA concentrations ≥10 μM. This pattern was not observed for BWA4C in the present study, and we are not aware of other 5-LO inhibitors that show such characteristics. Possibly, AA at concentrations ≥10 μM positively modulates 5-LO activity at a second, stimulating AA-binding site (Aharony and Stein, 1986; Hammarberg *et al.*, 2001; Burkert *et al.*, 2003) and this might be effectively blocked by HZ52. Alternatively, at concentrations ≥10 μM, AA may form micelles or lipid vesicles that favour the incorporation of HZ52 and thus allow more efficient access of the inhibitor towards 5-LO. Interestingly, in intact PMNL, HZ52 consistently inhibited 5-LO with IC_{50} = 0.4 to 1 μM up to 40 μM AA, indicating that the AA-dependent, distinct high and low potencies of HZ52 in the cell-free assay are not evident in intact cells. Together, our data indicate that HZ52 does not act by classical competition with AA as substrate at the active site but it may interfere with an allosteric site affected by AA.

In conclusion, our data reveal HZ52 to be a novel type of selective and effective 5-LO inhibitor *in vitro* and *in vivo* with marked efficacy in animal models of diseases that involve LTs. Importantly, HZ52 does not act as redox-active agent or iron-ligand inhibitor and does not exhibit the disadvantages of nonredox-type 5-LO inhibitors, such as loss of potency at elevated oxidative tone or strong dependency on the stimulus. These features confer on HZ52 great potential as a promising lead candidate for further preclinical assessment as a pharmacological agent for the treatment of LT-related disorders.

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Conflicts of interest

None.

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