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BRP-187: A potent inhibitor of leukotriene biosynthesis that acts through impeding the dynamic 5-lipoxygenase/5-lipoxygenase-activating protein (FLAP) complex assembly

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ABSTRACT

The pro-inflammatory leukotrienes (LTs) are formed from arachidonic acid (AA) in activated leukocytes, where 5-lipoxygenase (5-LO) translocates to the nuclear envelope to assemble a functional complex with the integral nuclear membrane protein 5-LO-activating protein (FLAP). FLAP, a MAPEG family member, facilitates AA transfer to 5-LO for efficient conversion, and LT biosynthesis critically depends on FLAP. Here we show that the novel LT biosynthesis inhibitor BRP-187 prevents the 5-LO/FLAP interaction at the nuclear envelope of human leukocytes without blocking 5-LO nuclear redistribution. BRP-187 inhibited 5-LO product formation in human monocytes and polymorphonuclear leukocytes stimulated by lipopolysaccharide plus *N*-formyl-methionyl-leucyl-phenylalanine (IC_{50} = 7–10 nM), and upon activation by ionophore A23187 (IC_{50} = 10–60 nM). Excess of exogenous AA markedly impaired the potency of BRP-187. Direct 5-LO inhibition in cell-free assays was evident only at >35-fold higher concentrations, which was reversible and not improved under reducing conditions. BRP-187 prevented A23187-induced 5-LO/FLAP complex assembly in leukocytes but failed to block 5-LO nuclear translocation, features that were shared with the FLAP inhibitor MK886. Whereas AA release, cyclooxygenases and related LOs were unaffected, BRP-187 also potently inhibited microsomal prostaglandin E_2 synthase-1 (IC_{50} = 0.2 μM), another MAPEG member. *In vivo*, BRP-187 (10 mg/kg) exhibited significant effectiveness in zymosan-induced murine peritonitis, suppressing LT levels in peritoneal exudates as well as vascular permeability and neutrophil infiltration. Together, BRP-187 potently inhibits LT biosynthesis *in vitro* and *in vivo*, which seemingly is caused by preventing the 5-LO/FLAP complex assembly and warrants further preclinical evaluation.

Abbreviations: 5-LO, 5-lipoxygenase; AA, arachidonic acid; COX, cyclooxygenase; $cPLA_2$, cytosolic phospholipase A_2; FLAP, 5-lipoxygenase-activating protein; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; H(P)ETE, hydro(pero)oxyeicosatetraenoic acid; HHT, hydroxyheptadecatrienoic acid; IL, interleukin; LPS, lipopolysaccharide; LT, leukotriene; mPGES-1, microsomal prostaglandin E_2 synthase 1; MPO, myeloperoxidase; PG, phosphate-buffered saline plus 1 mg/ml glucose; PGC, phosphate-buffered saline plus 1 mg/ml glucose and 1 mM $CaCl_2$; PMNL, polymorphonuclear leukocytes.

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1. Introduction

Leukotrienes (LT) are pro-inflammatory lipid mediators derived from arachidonic acid (AA) and formed via the 5-lipoxygenase (5-LO) pathway [1]. Upon activation of leukocytes, AA is released by cytosolic phospholipase ($cPLA_2$) and converted to 5(S)-hydroper oxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HPETE) in a first-step catalysis of 5-LO. In a second 5-LO-mediated reaction, 5-HPETE is then converted to the unstable epoxide LTA_4, a common precursor that can be transformed by LTC_4 synthases to the bronchoconstrictive cysteinyl-leukotrienes (cysLTs) or by LTA_4 hydrolase to the chemoattractant LTB_4 [2]. Due to the pro-inflammatory action of LTs, inhibitors against the enzymes in the LT biosynthetic pathway (i.e., 5-LO, LTA_4 hydrolase, LTC_4 synthases) [3–5] or compounds that act as antagonists at LT receptors [6] have been developed. However, besides CysLT1 receptor antagonists such as montelukast or pranlukast that are clinically used to treat (childhood) asthma, inhibitors against biosynthetic enzymes within the 5-LO pathway are not widely used in the clinics, certainly due also to significant side effects like liver toxicity [7]. As a consequence, an unmet need to develop new anti-LT compounds remains.

A promising target that is involved in the biosynthesis of the precursor LTA_4 is the 5-LO-activating protein (FLAP), which *in vivo* is seemingly indispensable for LT formation [8,9]. FLAP, a member of the MAPEG family (membrane-associated proteins in eicosanoid and glutathione metabolism), is an integral nuclear membrane protein lacking enzymatic activity but it is playing a crucial role in LT formation in various intact leukocytes [8,10]. Upon activation, 5-LO redistributes from soluble cellular pools to the perinuclear region and interacts with FLAP at the nuclear membrane [11,12]. FLAP facilitates AA substrate transfer to 5-LO [13,14] and AA is required for assembling the 5-LO/FLAP complex. In fact, FLAP inhibitors efficiently inhibit 5-LO product formation in intact cells which can be overcome, at least in part, by supplementation of high concentrations of exogenous AA [9,11,14–16]. Notably, FLAP inhibitors fail to inhibit 5-LO activity in cell-free assays [17,18], implying that FLAP is operative in 5-LO product biosynthesis only in the cellular context. The crystal structure of FLAP provided substantial insights into the binding site of AA and also clarified how FLAP inhibitors (i.e., MK591) might compete with AA from binding to FLAP [19]. Moreover, we recently showed that FLAP may help to coordinate 5-LO membrane binding with unblocking active AA-binding site access that is corked by Y181 and F177 [14]. Together, a dynamic 5-LO/FLAP complex is crucial for LTA_4 biosynthesis and represents, besides direct targeting of 5-LO enzymatic activity, a valuable target for pharmacological inhibitors in order to effectively and selectively intervene with LT-related disorders.

Within the frame of a recent screening approach for novel chemotypes that antagonize FLAP using a combined ligand- and structure based pharmacophore model [20], compound **1** (2-[4-(4 -chlorophenyl)-3-methyl-1,2-oxazol-5-yl)-5-[(2-methylphenyl)m ethoxy)phenol, Fig. 1A was identified as moderate inhibitor of 5-LO product biosynthesis in human neutrophils (IC_{50} = 4.4 μM), with unclear mode of action. In a follow-up study aiming to improve

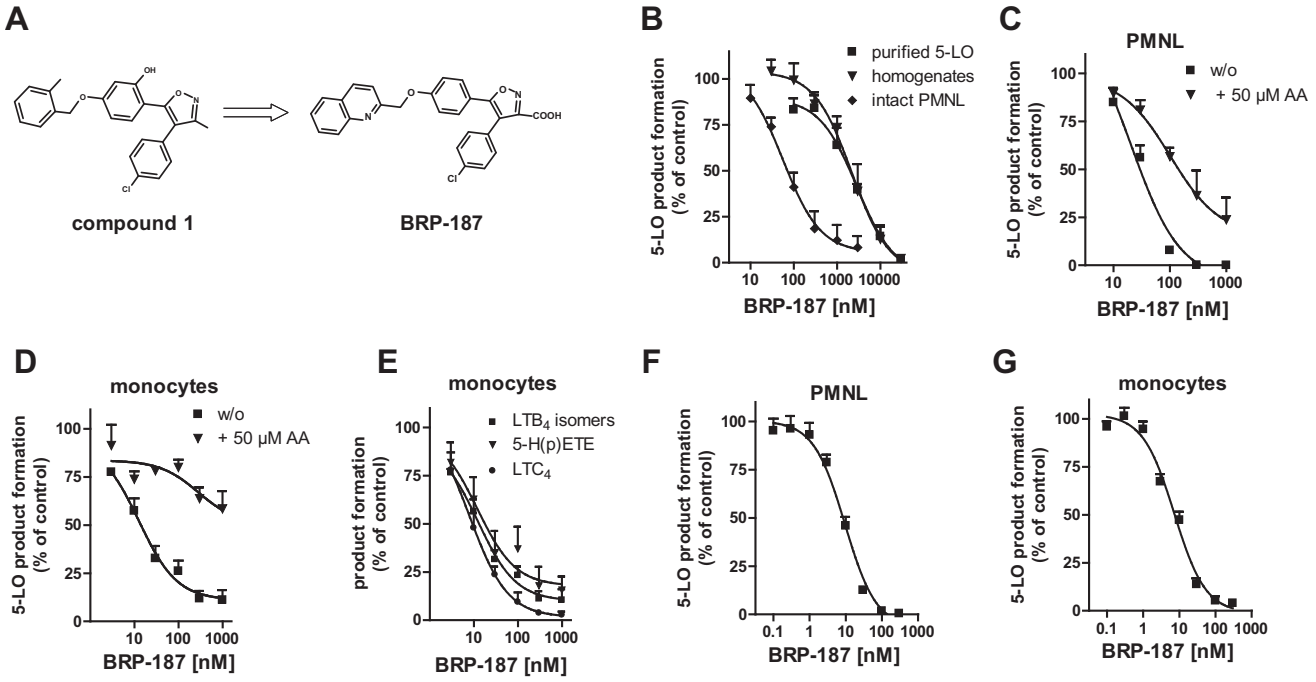

Fig. 1. BRP-187 potently inhibits 5-LO product formation in human PMNL and monocytes. (A) Chemical structures of parental compound 1 (2-[4-(4-chlorophenyl)-3-methyl-1,2-oxazol-5-yl)-5-[(2-methyl phenyl)methoxy)phenol), and the novel derivative BRP-187 (4-(4-chlorophenyl)-5-[4-(quinolin-2-ylmethoxy)phenyl]isoxazol-3-carboxylic acid). (B) Inhibition of 5-LO activity by BRP-187 in cell-based and cell-free assays. Human PMNL (5×10^6) were pre-incubated with BRP-187 or 0.1% DMSO as vehicle for 15 min at 37 °C prior stimulation by 2.5 μM A23187. After 10 min, 5-LO product formation was assessed. Purified 5-LO (0.5 μg/ml) or PMNL homogenates (corresponding to 5×10^6 cells/ml) were incubated with BRP-187 or vehicle (DMSO, 0.1%) at 4 °C for 15 min, samples were pre-warmed for 30 s at 37 °C, and 2 mM $CaCl_2$ and 20 μM AA were added. After 10 min 5-LO product formation was determined. (C–G) Human PMNL (C, F) or monocytes (D, E, G) were pre-incubated with BRP-187 or 0.1% DMSO as vehicle for 15 min at 37 °C. Cells were then stimulated for 10 min with 2.5 μM A23187 with or without 50 μM AA (C, D), or first primed with 1 μg/ml LPS for 20 min at 37 °C and then stimulated with 1 μM fMLP for 10 min (F, G). (E) Inhibition of single 5-LO products formed in A23187-activated monocytes including trans-isomers of LTB_4, 5-HETE and LTC_4 by BRP-187, see (D). For the corresponding 100% control, typical ranges of 5-LO products formed are: LTB_4 and its isomers: 20–30 ng/10^6 cells; 5-H(P)ETE: 10–15 ng/10^6 cells; LTC_4: 0.5–0.7 ng/10^6 cells. In all assays, 5-LO products LTB_4, its trans-isomers, and 5-HETE were analyzed by HPLC, whereas LTC_4 was assessed by ELISA. Data are expressed as percentage of control (100%), mean ± SEM, n = 4.

the LT biosynthesis inhibitory potency of compound **1** through interference with FLAP, the derivative BRP-187 (4-(4-chlorophenyl)-5-[4-(quinolin-2-ylmethoxy)phenyl]isoxazol-3-carboxylic acid, Fig. 1A was obtained that potently inhibited cellular 5-LO product formation with an IC_{50} = 0.24 μM and >30-fold lower activity against 5-LO directly in cell-free assays [21]. Here we (i) provide a comprehensive analysis of BRP-187 on 5-LO product biosynthesis *in vitro* and *in vivo*, (ii) reveal that this compound impedes the 5-LO/FLAP complex assembly, and (iii) demonstrate potent anti-inflammatory effectiveness of BRP-187 in a LT-related animal model of inflammation.

2. Materials and methods

2.1. Materials

BRP-187 was synthesized and characterized as previously described by us [21]. Zileuton (*N*-(1-benzo[b]thien-2-ylethyl)-*N*-hydroxyurea), Sequoia Research Products (Oxford, UK); MK886, LTC_4-d5 methyl ester and LTA_4-methyl ester, Cayman Chemicals (Ann Arbor, MI); PGH_2, Larodan (Malmö, Sweden); IL-1β, Repro-Tech (Hamburg, Germany); pyrrolidine-1, Calbiochem (Darmstadt, Germany); EDTA and Nonidet P-40, AppliChem (Darmstadt, Germany); RPMI media, glutamine, penicillin and streptomycin, PAA (Coelbe, Germany); tritium-labeled [5,6,8,9,11,12,14,15-3H]AA, Biotrend Chemicals GmbH (Cologne, Germany), Adenosine deaminase (Ada), AA, ATP-agarose, Ca^{2+}-ionophore A23187, dextran, dithiothreitol, fetal calf serum, formyl-methionyl-leucyl-phenylalanine (fMLP), indomethacin, lipopolysaccharide (LPS), Triton X-100 and all other chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany), unless stated otherwise. HPLC solvents were from VWR (Darmstadt, Germany).

2.2. Cells and cell isolation

Peripheral blood (University Hospital Jena, Germany) was withdrawn from fasted (12 h) healthy adult volunteers that had not taken any anti-inflammatory drugs during the last 10 days (with consent), by venipuncture in heparinized tubes (16 IE heparin/ml blood). The blood was then centrifuged (4000*g*, 20 min, 20 °C) for preparation of leukocyte concentrates. Leukocyte concentrates were subjected to dextran sedimentation and centrifugation on lymphocyte separation medium (LSM 1077, PAA, Colbe, Germany). For isolation of polymorphonuclear leukocytes (PMNL), contaminating erythrocytes of pelleted PMNL were removed by hypotonic lysis. PMNL were then washed twice in ice-cold PBS and finally resuspended in PBS pH 7.4 containing 1 mg/ml glucose or in PBS pH 7.4 containing 1 mg/ml glucose plus 1 mM $CaCl_2$ (PGC buffer) (purity >96–97%). Monocytes were isolated from the peripheral blood mononuclear cell fraction, which was obtained after centrifugation of leukocyte concentrates on LSM 1077, by adherence (1.5 h at 37 °C) to culture flasks (Greiner, Nuertingen, Germany) at a cell density of 2×10^7 cells/ml RPMI 1640 medium containing 2 mM L-glutamine and 100 U/ml penicillin and 100 μg/ml streptomycin). The purity of monocytes was >85%, defined by forward- and side-light scatter properties and detection of the CD14 surface molecule by flow cytometry (BD FACS Calibur, Heidelberg, Germany). Monocytes were finally resuspended in ice-cold PGC buffer for further experiments. For analysis of acute cytotoxicity of BRP-187 during pre-incubation (i.e. 30 min), the viability of PMNL and monocytes was analyzed by trypan blue exclusion using a Vi-cell counter (Beckmann Coulter, Krefeld, Germany).

HEK293 and A549 cells used as source for expression of LTC_4 synthase and mPGES-1 were obtained at LGC Standards (Wesel, Germany). Authentication of cell lines was carried out by STR analyses (LGC Standards).

2.3. Expression, purification and activity assay of human recombinant 5-LO

Escherichia coli (BL21) was transformed with pT3-5-LO plasmid, and recombinant 5-LO protein was expressed at 30 °C as described [22]. Cells were lysed in 50 mM triethanolamine/HCl pH 8.0, 5 mM EDTA, 1 mM phenylmethanesulphonyl fluoride, soybean trypsin inhibitor (60 μg/ml), and lysozyme (1 mg/ml), homogenized by sonication (3 × 15 s), and centrifuged at 40,000*g* for 20 min at 4 °C. The 40,000*g* supernatant (S40) was applied to an ATP-agarose column to partially purify 5-LO as described [22]. Aliquots of semi-purified 5-LO were diluted with ice-cold PBS containing 1 mM EDTA, and 1 mM ATP was added. Samples were pre-incubated with the test compounds or vehicle (0.1% DMSO). After 10 min at 4 °C, samples were pre-warmed for 30 s at 37 °C, and 2 mM $CaCl_2$ plus the indicated concentrations of AA were added to start 5-LO product formation. The reaction was stopped after 10 min by addition of one volume of ice-cold methanol, and the formed metabolites were analyzed by RP-HPLC as described [23]. 5-LO products include the all-trans isomers of LTB_4 as well as 5(*S*)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HPETE) and its corresponding alcohol 5(*S*)-hydroxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HETE).

2.4. Determination of 5-lipoxygenase products in intact cells and in corresponding homogenates

For determination of 5-LO products in intact PMNL (5×10^6) or monocytes (2×10^6) cells were resuspended in 1 ml PGC buffer, preincubated for 15 min at 37 °C with test compounds or vehicle (0.1% DMSO), and incubated for 10 min at 37 °C with the indicated stimuli. Ca^{2+}-ionophore A23187 (2.5 μM) was added with or without the indicated concentrations of AA for 10 min and the reaction was stopped on ice by addition of 1 ml of methanol. Alternatively, cells were first primed at 37 °C with LPS (1 μg/ml) plus Ada (0.3 U/ml) for 30 min. Formation of 5-LO products was started by addition of 1 μM fMLP and after 5 min the reaction was stopped by methanol. 30 μl 1 N HCl and 500 μl PBS, and 200 ng prostaglandin B_1 were added and the samples were subjected to solid phase extraction on C18-columns (100 mg, UCT, Bristol, PA, USA). 5-LO products (LTB_4 and its trans-isomers, 5-H(P)ETE), were analyzed by HPLC and quantities calculated on the basis of the internal standard PGB_1. CysLTs C_4, D_4 and E_4 were not detected (amounts were below detection limit), and oxidation products of LTB_4 were not determined.

For determination of 5-LO products in corresponding homogenates, cell suspensions were resuspended in PBS containing 1 mM EDTA for 5 min at 4 °C and sonicated (4 × 10 s, 4 °C). Homogenates corresponding to 5×10^6 cells/ml were incubated with the test compounds or vehicle (0.1% DMSO) for 15 min at 4 °C, pre-warmed for 30 s at 37 °C and the reaction was started by the addition of 2 mM $CaCl_2$ and 20 μM AA. The reaction was stopped after 10 min and the samples were analyzed as described for intact cells above.

2.5. Determination of cysLT formation in monocytes

Monocytes (5×10^6 cells) were resuspended in 1 ml PGC buffer, incubated with the test compounds or vehicle (0.1% DMSO) for 15 min at 37 °C, primed with 1 μg/ml LPS for 20 min and stimulated with 1 μM fMLP for 10 min at 37 °C. The reaction was stopped on ice, and supernatants were collected after centrifugation at 600*g* for 10 min at 4 °C. CysLTs were assessed by ELISA,

which detects LTC_4, LTD_4 and LTE_4 according to the manufacturer's (Enzo Life Sciences International Inc., Lörrach, Germany) instructions.

2.6. Analysis of 5-LO redistribution by immunofluorescence microscopy (IF)

For analysis of 5-LO and FLAP distribution in monocytes, PBMC were seeded in RPMI medium containing 2 mM L-glutamine and 100 U/ml penicillin and 100 μg/ml streptomycin onto glass coverslips in a 12-well plate and cultured for 1.5 h. Cells were washed with PGC buffer and pre-incubated with test compounds or vehicle 10 min at 37 °C in PGC buffer prior to activation. For analysis of PMNL, cells were pre-incubated with test compounds or vehicle for 10 min at 37 °C resuspended in PGC buffer and centrifuged onto poly-D-lysine coated glass coverslips (10*g* for 2 s) prior to stimulation. Monocytes and PMNL were then activated for 3 min and stopped by fixation with 4% paraformaldehyde solution. Acetone (3 min, 4 °C) and 0.25% Triton X-100 (10 min) were used for permeabilization prior to blocking with non-immune goat serum. Samples were incubated with mouse monoclonal anti-5-LO antibody (1:100; generous gift by Dr. Dieter Steinhilber (Goethe University Frankfurt, Germany) and rabbit polyclonal anti-FLAP antibody (5 μg/ml; Abcam (Cambridge, UK) at 4 °C [9]. 5-LO and FLAP were stained with the fluorophore-labeled secondary antibodies; Alexa Fluor 488 goat anti-rabbit (1:1000) and Alexa Fluor 555 goat anti-mouse (1:1000), obtained at Invitrogen (Darmstadt, Germany) [9]. Nuclear DNA was stained with DAPI (Invitrogen). Samples were analyzed by a Zeiss Axiovert 200 M microscope, and a Plan Neofluar ×100/1.30 Oil (DIC III) objective (Carl Zeiss, Jena, Germany). An AxioCam MR camera (Carl Zeiss, Jena, Germany) was used for image acquisition

2.7. Determination of PGE_2 synthase activity in a cell-free assay

Preparations of A549 cells and determination of mPGES-1 activity was performed as described previously [24]. In brief, A549 cells were treated with 2 ng/ml Il-1β for 72 h at 37 °C, 5% CO_2. Cells were harvested, sonicated and homogenized. The homogenate was subjected to differential centrifugation at 10,000*g* for 10 min and 174,000*g* for 1 h at 4 °C. The pellet (microsomal fraction) was resuspended in 1 ml homogenization buffer. Microsomal membranes were diluted in potassium phosphate buffer (0.1 M, pH 7.4) containing 2.5 mM glutathione. Test compounds or vehicle were added, and after 15 min at 4 °C reaction (100 μl total volume) was initiated by addition of 20 μM PGH_2. After 1 min at 4 °C, the reaction was terminated using stop solution (100 μl; 40 mM $FeCl_2$, 80 mM citric acid, and 10 μM 11β-PGE_2 as internal standard). PGE_2 was separated by solid-phase extraction and analyzed by RP-HPLC as described previously [24].

2.8. Determination of LTC_4 synthase activity

HEK293 cells stably expressing LTC_4 synthase (HEK_LTC_4-S) were used to generate microsomes containing LTC_4 synthase. Briefly, HEK_LTC4-S were frozen in liquid nitrogen and sonicated 3 × 20 s at 4 °C in homogenization buffer (0.1 potassium phosphate buffer, pH 7.4, 1 mM phenylmethylsulfonylfluoride, 60 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin, 2.5 mM glutathione, and 250 mM sucrose). Lysates were sequentially centrifuged at 10,000*g*, 10 min at 4 °C and 174,000 × *g* for 1 h at 4 °C. The microsomal fraction (2.5 μg) was resuspended in assay buffer (0.1 M potassium buffer pH 7.4; plus 5 mM glutathione) and preincubated with the test compound or vehicle (0.1% DMSO) for 10 min at 4 °C. The reactions were started by adding 1 μM LTA_4-methyl ester (Cayman, Ann Arbor, MI) and stopped after 10 min incubation at 4 °C by 1 vol. ice-cold methanol. Acidified PBS and LTC_4-methyl ester-d_5 as internal standard were added prior solid phase extraction and LTC_4 methyl ester formation was analyzed by UPLC–MS/MS as described [25]. UPLC–MS/MS was carried out on an Acquity UPLC BEH C18 column (1.7 μm, 2.1 × 50 mm, waters) and a QTRAP 5500 Mass spectrometer (AB Sciex). LTC_4 methyl ester and LTC_4-d5 methyl ester (standard) were detected by multiple reaction monitoring in the negative ion mode by a method described previously [26].

2.9. Analysis of protein interaction by in situ proximity ligation assay

To analyze the *in situ* interaction of 5-LO with FLAP in monocytes and PMNL, an *in situ* proximity ligation assay (PLA) was performed, according to the manufacturers' protocol [27] and as described [11]. Samples were treated, fixed and incubated with primary antibody as described for IF microscopy above. Cells were then incubated with species specific secondary antibodies conjugated with oligonucleotides (PLA probe anti-mouse MINUS and anti-rabbit PLUS) for 1 h at 37 °C. By addition of two other circle-forming DNA oligonucleotides and a ligase (30 min at 37 °C) the antibody-bound oligonucleotides form a DNA circle when the target proteins are less than 40 nm distant from each other. The newly generated DNA circle was amplified by rolling circle amplification and visualized by hybridization with fluorescently labeled oligonucleotides. Nuclear DNA was stained with DAPI. The PLA interaction signal appears as a fluorescent spot and was analyzed by fluorescence microscopy using a Zeiss Axiovert 200 M microscope, and a Plan Neofluar ×100/1.30 Oil (DIC III) objective as well as a Plan Neofluar ×40/1.30 Oil (DIC III) objective (Carl Zeiss, Jena, Germany).

2.10. Determination of release of [^{3}H]-labeled arachidonic acid

Release of [^{3}H]-labeled AA from human monocytes was analyzed as described [28]. Briefly, cells were resuspended in medium and incubated with 5 nM [^{3}H]AA (corresponding to 0.5 μCi/ml, specific activity 200 Ci/mmol) for 2 h at 37 °C. Cells were collected, washed to remove unincorporated [^{3}H]AA, and resuspended in PBS containing 1 mM $CaCl_2$. The cells were pre-incubated with test compounds or vehicle (0.1% DMSO) at 37 °C for 15 min, and subsequently stimulated with 2.5 μM A23187 for 10 min at 37 °C. The reaction was stopped on ice (10 min) and cells were centrifuged. Aliquots of the supernatants were combined with 2 ml Rotiszint® eco plus and assayed for radioactivity by scintillation counting (Micro Beta Trilux, Perkin Elmer, Waltham, MA).

2.11. Murine peritonitis model

Male CD-1 mice (33–39 g, Charles River Laboratories, Calco, Italy) were housed in a controlled environment (21 ± 2 °C) and provided with standard rodent chow and water ad libitum. Mice were allowed to acclimate for five days prior to experiments and were kept at 12 h light–12 h dark schedule. Experiments were conducted during the light phase. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (Ministerial Decree 116/92) and with the European Economic Community regulations (Official Journal of E.C. L 358/1 12/18/1986). Animal studies were approved by the local ethics committee of the Univ. of Naples Federico II on 27 February 2014 (approval number 2014/18760).

Mice (n = 6 per experimental group; age of 8–9 weeks) received BRP-187 (1, 3 or 10 mg/kg), MK886 (1 or 3 mg/kg), zileuton (10 mg/kg) or vehicle (0.5 ml of 0.9% saline solution containing 2% DMSO), intraperitoneally (i.p.) 30 min prior to zymosan injection, according to a well-recognized experimental design for

studying LT synthesis inhibitors in acute inflammation [29,30]. After boiling in PBS and centrifugation, zymosan (Sigma, Milan, Italy) was prepared as a final suspension (2 mg/ml) in 0.9% (w/v) saline and injected i.p. (0.5 ml) after sonication. Mice were killed by inhalation of CO_2 at the indicated time points followed by a peritoneal lavage with 3 ml of cold PBS. After 60 s gentle manual massage, 2 ml of exudates were collected, and infiltrated cells were counted with a light microscope in a Burker's chamber after vital trypan blue staining. The samples were centrifuged (18,000*g*, 5 min, 4 °C) and frozen (-80 °C) for the myeloperoxidase (MPO) assay (pellet) and for cysLT analysis (supernatant). The amounts of cysLTs were assayed in the supernatant by EIA (Enzo Life Sciences International Inc., Lörrach, Germany) according to manufacturer's protocol; data are expressed as ng/ml.

MPO assay was carried out according to Rao et al. [30] with minor modifications. Briefly, pellets from exudates were resuspended into 1.7 ml PBS (50 mM; pH6) containing 0.5% hexadecyltrimethyl-ammonium bromide. To disrupt the cells, the samples were sonicated, followed by a freeze-thawing for 3 times and a second sonication step. After centrifugation (18,000*g*; 30 min) 20 µl of the supernatant was added to a 96-well plate. The reaction was initiated by addition of 200 µl PBS (50 mM; pH6) containing 0.167 mg/ml of *o*-dianisidine and 0.0005% hydrogen peroxide. The rate of change of absorbance was monitored in kinetic mode using a plate reader (Biorad Imark microplate). Levels of MPO were determined from the calibration curve using human neutrophil MPO as reference standard. MPO levels were expressed as units MPO per mouse.

Vascular permeability was assessed according to Kolaczkowska et al. [31] with minor modifications. In brief, Evans blue dye was solubilized in saline (5 mg/ml), diluted to give a constant dose of 40 mg/kg per mice (6 mice per group, with age of 8–9 weeks) and injected intravenously (i.v.) into the caudal vein (0.3 ml), immediately followed by peritonitis induction. After 30 min the mice were euthanized by CO_2 and exudates were collected after peritoneal lavage with 3 ml of cold PBS. After centrifugation at 3000*g* for 5 min, the absorbance of the supernatants was measured at 650 nm (Beckman Coulter DU730).

2.12. Statistics

Data are expressed as mean ± S.E.M. IC_{50} values were calculated from averaged measurements at 3–5 different concentrations of the compounds by nonlinear regression using GraphPad Prism software (San Diego, CA) one site binding competition. Data obtained in animal experiments are given as mean ± SEM obtained from 6 mice in total. 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.

3. Results

3.1. BRP-187 potently inhibits cellular 5-LO product synthesis

In agreement with the results from our recent SAR study on 4,5-diarylisoxazole-3-carboxylic acids [21], BRP-187 potently repressed 5-LO product biosynthesis in intact A23187-activated PMNL in a concentration-dependent manner, with IC_{50} = 60 nM (Fig. 1B). However, BRP-187 was 35- and 37-fold less efficient in inhibiting isolated 5-LO enzyme (IC_{50} = 2.1 µM) or 5-LO in crude in homogenates (IC_{50} = 2.2 µM), respectively, assayed in cell-free test systems (Fig. 1B). The higher potency of BRP-187 in intact cells versus cell-free assays implied that besides 5-LO, the compound may interfere with additional cellular determinants of 5-LO product biosynthesis such as $cPLA_2$ or FLAP. Therefore, we investigated suppression of 5-LO product biosynthesis in intact cells in more detail by variation of the cell-based assay conditions. First, we supplemented PMNL with exogenous AA in excess (50 µM) to circumvent $cPLA_2$-mediated AA substrate supply and the absolute requirement of FLAP [16,32,33]. As shown in Fig. 1C, also in the presence of 50 µM AA, BRP-187 still suppressed 5-LO activity in PMNL, but the IC_{50} shifted to 10-fold higher values (i.e., 0.4 µM).

Besides PMNL, we studied 5-LO activity also in human monocytes. BRP-187 strongly suppressed 5-LO product biosynthesis in A23187-activated monocytes (IC_{50} = 10 ± 4 nM) with significant higher potency than in PMNL, and again supplementation of 50 µM AA caused >100-fold loss of potency (IC_{50} > 1 µM) (Fig. 1D). Note that BRP-187 suppressed the synthesis of all 5-LO products (i.e., LTB_4, its trans-isomers, and 5-H(P)ETE) almost equally well (Fig. 1E). As monocytes produce also cysLTs as 5-LO products, we analyzed the effect of BRP-187 for repression of cysLT formation in A23187-treated cells (monitored by ELISA), and a similar concentration-dependent inhibition as for other 5-LO products with IC_{50} = 9 ± 2 nM was observed (Fig. 1E). Of interest, upon stimulation with the physiological relevant receptor-mediated agonists LPS and fMLP [34], BRP-187 was most potent and blocked LTB_4 formation in PMNL or monocytes with IC_{50} values of 7 ± 1 and 10 ± 1 nM, respectively (Fig. 1F and G). The IC_{50} values of BRP-187 are summarized in Table 1.

3.2. BRP-187 barely alters 5-LO subcellular redistribution but disrupts the interaction of 5-LO with FLAP

FLAP as an integral nuclear membrane protein may associate with 5-LO after redistribution from the cytosol to the perinuclear region upon cell activation [11,12]. FLAP inhibitors are able to interrupt this interaction, which can be visualized *in situ* by PLA [11]. 5-LO redistribution and colocalization with FLAP at the nuclear membrane can be studied by IF microscopy [9,11], but inconsistent observations were made concerning the effects of FLAP inhibitors that somehow alter 5-LO subcellular localization but do not unequivocally prevent translocation of 5-LO to the nucleus [9,11,15,29].

We first analyzed the effects of BRP-187 on 5-LO subcellular localization in human monocytes and PMNL, where colocalisation of 5-LO and FLAP at the nuclear membrane in A23187-activated cells can be clearly visualized by immunofluorescence (IF) microscopy (Fig. 2A). The 5-LO inhibitor zileuton [35] and the FLAP inhibitor MK886 [36] were used as negative and positive controls, respectively [11]. In activated monocytes and PMNL both MK886 and BRP-187 failed to prevent movement of 5-LO to the perinuclear region (Fig. 2A). Zileuton, used as negative control, did not affect 5-LO subcellular redistribution as expected and shown in our recent study before [11]. However, using PLA in human monocytes and PMNL, BRP-187 (0.1 µM) prevented the interaction between 5-LO and FLAP at the nuclear membrane in activated cells, and a comparable effect was obvious for cells treated with MK886

Table 1
Overview of the IC_{50} values of BRP-187 for 5-LO product formation in various test systems and assays.

Test system	Assay condition	IC_{50} [µM]
Isolated 5-LO	10 µM AA	2.1 ± 1.7
Homogenates (PMNL)	20 µM AA	2.2 ± 0.07
Intact PMNL	A23187	0.06 ± 0.01
	A23187 + 50 µM AA	0.4 ± 0.2
	LPS/fMLP	0.01 ± 0.001
Intact monocytes	A23187	0.01 ± 0.004
	A23187 + 50 µM AA	>1
	LPS/fMLP	0.007 ± 0.001

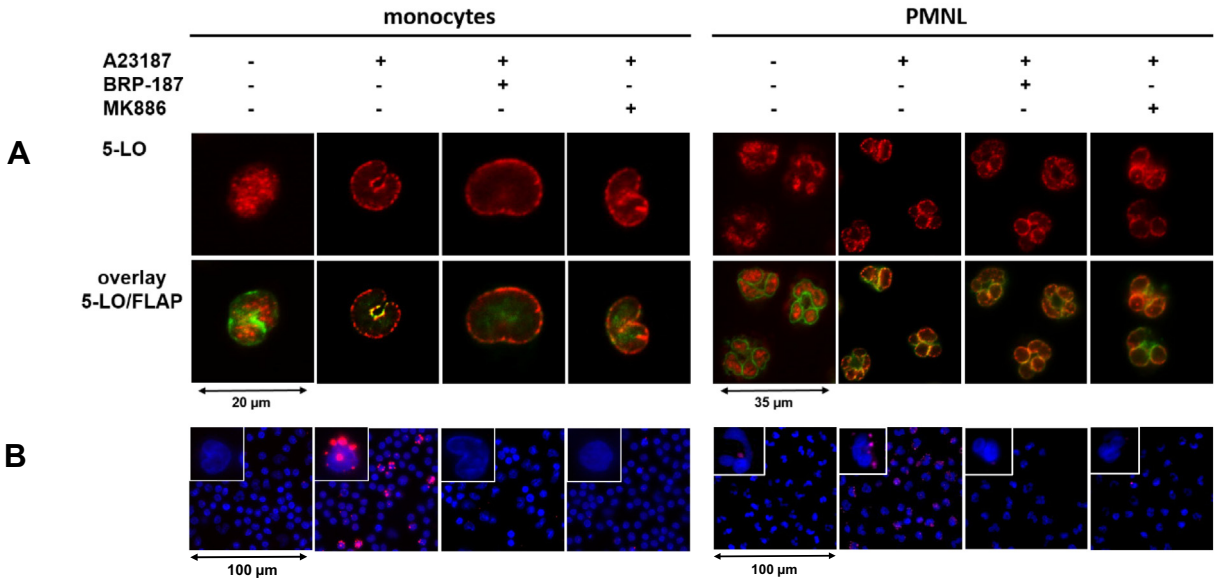

Fig. 2. Effects of BRP-187 on 5-LO subcellular redistribution and 5-LO/FLAP interaction. (A) Subcellular localization of 5-LO and FLAP. Monocytes (left panel) or PMNL (right panel) were pre-incubated with BRP-187 (1 µM), MK886 (300 nM) or vehicle (0.1% DMSO) for 10 min at 37 °C, and then stimulated with 2.5 µM A23187 for 10 min at 37 °C. Images show single staining for 5-LO (red, top lane) and overlay of 5-LO (red) and FLAP (green) (bottom lane). Results are representative for 100 individual cells of three independent experiments. (B) 5-LO/FLAP interaction. *In situ* PLA, using proximity probes against 5-LO and FLAP was performed in monocytes (left panel) and PMNL (right panel) pre-incubated with BRP-187 (100 nM) or MK886 (100 nM) for 15 min at 37 °C and stimulated with A23187 (2.5 µM) for 20 min. DAPI (blue) was used to stain the nucleus and PLA signals (magenta dots) visualize 5-LO/FLAP interaction. Results are representative for 100 individual cells analyzed in three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 2B), while again zileuton failed in this respect, as expected and reported before [11]. Together, BRP-187 seemingly acts as FLAP inhibitor and the potent suppression of 5-LO product biosynthesis in intact cells from endogenous AA might be due to prevention of 5-LO/FLAP complex formation.

3.3. Effects of BRP-187 on 5-LO activity in cell-free assays

We next analyzed the effects of BRP-187 on 5-LO activity in cell-free assays in more detail. Wash-out experiments using isolated 5-LO revealed a complete reversibility of 5-LO inhibition by BRP-187 (Fig. 3A). Also, when 5-LO inhibition by BRP-187 was investigated in the presence of 0.01 or 0.1% Triton X-100, a non-ionic detergent that should solubilize aggregates that may be formed due to the lipophilic nature of BRP-187, the potency was not or only hardly reduced (Fig. 3B). Thus, we conclude that inhibition of 5-LO by BRP-187 is caused by specific, reversible binding to the 5-LO enzyme, albeit with apparent low affinity requiring high IC_{50} values as compared to the potent repression of 5-LO product synthesis in intact cells.

As for BRP-187, for nonredox-type (competitive) 5-LO inhibitors such as CJ-13,610 or ZM230487, the potency is also high in intact cells but low in cell-free assays [37,38]. However, reducing conditions (e.g., presence of glutathione peroxidase and thiols such as DTT) or low AA concentrations confer these inhibitors high efficiency for repression of 5-LO [37,38]. In contrast to nonredox-type 5-LO inhibitors [38], addition of 1 mM DTT to homogenates of PMNL the potency of BRP-187 was not significantly altered (Fig. 3C). Moreover, and again unlike nonredox-type (competitive) 5-LO inhibitors [38], lowering the AA concentration did not improve the potency of BRP-187 (not shown), excluding this compound as being a nonredox-type 5-LO inhibitor.

3.4. Direct targets of BRP-187 within the AA cascade

Since BRP-187 lost potency in A23187-activated PMNL and monocytes in the presence of exogenous AA, we analyzed if the compound blocked release of AA from endogenous sources, which (besides acting on FLAP) might be another reason for the high cellular potency. However, in [^{3}H]AA-prelabeled monocytes, BRP-187 even at 10 µM did not suppress the A23187-induced release of [^{3}H]AA, while the $cPLA_2$ inhibitor RSC-3388 (1 µM) markedly blocked it, as expected (Fig. 4A). Moreover, unspecific interference of BRP-187 with the viability of PMNL or monocytes can be excluded, as 30 min pre-incubation of the cells with 10 µM BRP-187 did not affect cellular integrity (analyzed by trypan blue exclusion, not shown) or metabolic activity over 24 h as monitored by MTT assay in monocytes (Fig. 4B, staurosporine as control).

Our previous study revealed no significant effects of BRP-187 (up to 10 µM) on 5-LO-related dioxygenases that transform AA such as 12/15-LOs, COX-1 and COX-2 [21]. However, the possibility that FLAP is the major target prompted us to investigate if FLAP-related enzymes out of the MAPEG family may be affected by BRP-187 as well, which is the case for the structurally unrelated FLAP inhibitor MK886 [39,40]. In fact, the activity of mPGES-1 that converts PGH_2 to PGE_2 was concentration-dependently inhibited by BRP-187 in a cell-free assay with an IC_{50} = 0.2 µM (Fig. 4D) which even clearly outperforms MK886 (i.e., IC_{50} = 2.4 µM [41]) that served as control. Similarly, the MAPEG member LTC_4 synthase was blocked by BRP-187 with IC_{50} = 6 µM, again MK886 was used as reference (IC_{50} approx. 3 µM [40]) (Fig. 4C). Together, BRP-187 prevents cellular 5-LO/FLAP complex assembly, thereby potently suppressing 5-LO product biosynthesis in the one-digit nanomolar range, but also directly inhibits the activity of the MAPEG members mPGES-1 and LTC_4 synthase as well as 5-LO at higher concentrations without affecting the 5-LO-related dioxygenases 12/15-LOs and COX-1/2.

3.5. Anti-inflammatory effectiveness of BRP-187 and inhibition of LT biosynthesis in vivo

In view of the high potency of BRP-187 for suppression of cellular LTB_4 and cysLT formation we attempted to evaluate the anti-inflammatory effectiveness and inhibition of LT biosynthesis

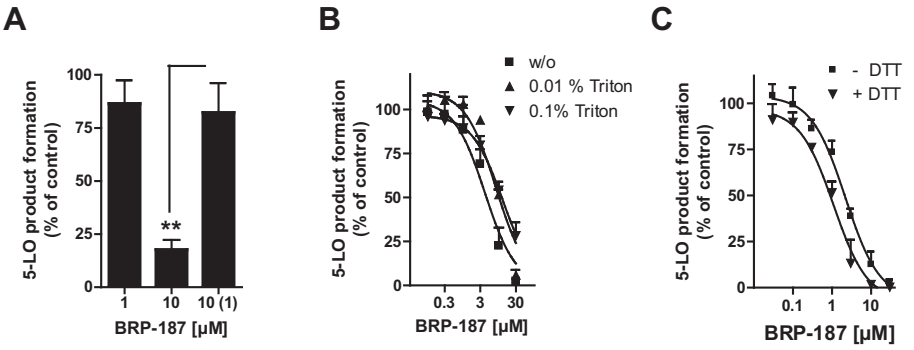

Fig. 3. BRP-187 inhibits 5-LO product formation in cell-free assays. (A) Reversible inhibition of 5-LO by BRP-187. Purified 5-LO (0.5 μg/ml) was incubated with 1 μM or 10 μM BRP-187 for 15 min at 4 °C. An aliquot of the 10 μM sample was 10-fold ("10 (1)") diluted with assay buffer, while the other aliquot was not altered. The samples were then prewarmed for 30 s at 37 °C and 2 mM $CaCl_2$ and 20 μM AA were added. After 10 min, 5-LO product formation was analyzed by HPLC. **, $p < 0.01$ versus inhibition without dilution, ANOVA + Bonferroni post hoc test; n = 3. (B) Purified 5-LO (0.5 μg/ml) was incubated with BRP-187 in the absence or presence of 0.01% or 0.1% triton-X 100 at 4 °C for 15 min, and 5-LO activity was determined as described above. Data are expressed as percentage of control (100%) and given as mean ± SEM, n = 4. (C) Effects of DTT on 5-LO inhibition by BRP-187. PMNL homogenates (corresponding to 5×10^6 cells/ml) were pre-incubated with BRP-187 or vehicle (DMSO, 0.1%) with or without 1 mM DTT at 4 °C for 15 min. Samples were then pre-warmed for 30 s at 37 °C, 2 mM $CaCl_2$ and 20 μM AA were added, and after 10 min 5-LO product formation was determined. Data are expressed as percentage of control (100%), mean ± SEM, n = 3.

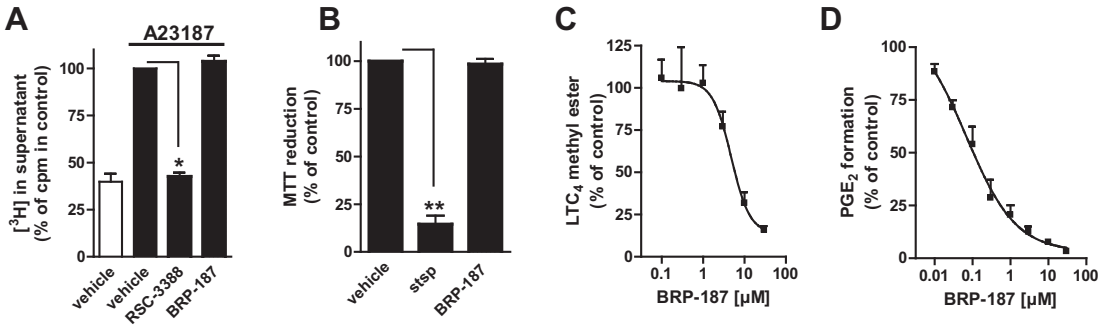

Fig. 4. Effects of BRP-187 on cellular AA release, cell viability, LTC4 synthase activity and mPGES-1 activity. (A) Cellular AA release. Analysis of radioactivity in supernatants of [^{3}H]AA-labeled monocytes treated with vehicle (0.1% DMSO) and after pre-incubation with 10 μM BRP-187 or 1 μM $cPLA_2$ inhibitor (RSC-3388) and stimulation with 2.5 μM A23187. Data are expressed as percentage of control (100%), mean ± SEM, n = 4. *, $p < 0.05$, versus vehicle control. (B) Cell viability. Monocytes were treated with BRP-187, staurosporine (stsp, 3 μM) or vehicle (0.3% DMSO) for 24 h and cell viability was analyzed by MTT assay. Data are expressed as percentage of control (100%), mean ± SEM, n = 3. **, $p < 0.005$ versus vehicle control. (C) LTC4 synthase activity. LTC_4 synthase-containing microsomes from transfected HEK293 cells were pre-incubated with BRP-187 or vehicle (0.1% DMSO) at 4 °C for 10 min. Then, samples were pre-warmed for 1 min at 37 °C and 1 μM LTA_4-methyl ester was added. After 10 min the reaction was stopped and LTC_4 methyl ester was analyzed. Data are expressed as percentage of control (100%), mean ± SEM, n = 3. (D) mPGES-1 activity. Microsomes of IL-1β-stimulated A549 cells were pretreated with BRP-187 treated at 4 °C for 10 min and 20 μM PGH_2 was added. After 1 min at 4 °C, the reaction was stopped and PGE_2 was analyzed. Data are expressed as percentage of control (100%), mean ± SEM, n = 4.

in vivo in the zymosan-induced mouse peritonitis model [30]. Mice (n = 6 per group) received i.p. BRP-187 (1, 3, or 10 mg/kg), MK886 (1 or 3 mg/kg), zileuton (10 mg/kg) or vehicle (2% DMSO in saline) 30 min prior to i.p. zymosan injection. Thirty min after zymosan, vascular permeability was analyzed and exudates were collected for measuring cysLT levels, and after 4 h, exudates were collected for analysis of leukocyte infiltration and MPO activity. BRP-187 dose-dependently reduced cysLT levels by 20% at 3 mg/kg and 60% at 10 mg/kg. MK886 (1 mg/kg) decreased cysLT levels by 72% and zileuton (10 mg/kg) by 35% (Table 2). BRP-187, at 10 mg/kg, also significantly attenuated (33%) the increased vascular permeability (Table 3), the infiltration of leukocytes (57%), and completely abolished MPO activity (Table 4). The FLAP reference inhibitor MK886 also prevented vascular permeability (51% at

Table 2
Effects of BRP-187 *in vivo* on cysLT levels in zymosan-induced peritonitis. Male mice (n = 6, each group) were treated i.p. with BRP-187, MK886, zileuton or vehicle, 30 min before i.p. injection of zymosan. Analysis of cysLT levels by ELISA was performed 30 min after zymosan injection. *$p < 0.05$; ***$p < 0.001$ versus vehicle (Anova + Bonferroni).

Treatment	CysLTs	
	ng/ml	% inhibition
Vehicle	87.3 ± 9.7	–
1 mg/kg BRP-187	81.7 ± 11.1	6%
3 mg/kg BRP-187	69.5 ± 9.9	20%
10 mg/kg BRP-187	34.6 ± 3.9***	60%
1 mg/kg MK886	24.2 ± 2.9***	72%
10 mg/kg zileuton	56.5 ± 4.6*	35%

Table 3
Effects of BRP-187 on vascular permeability in zymosan-induced peritonitis. Male mice (*n* = 6, each group) were treated i.p. with BRP-187, MK886, zileuton or vehicle, 30 min before i.p. injection of zymosan. Analysis of vascular permeability was performed 30 min after zymosan injection. *$p < 0.05$; ***$p < 0.001$ versus vehicle (*t*-test); n.i., no inhibition.

Treatment	Vascular permeability	
	A_{610}	% inhibition
Vehicle	0.623 ± 0.035	–
10 mg/kg BRP-187	0.417 ± 0.071*	33%
3 mg/kg MK886	0.303 ± 0.018***	51%
10 mg/kg zileuton	0.639 ± 0.104	n.i.

Table 4
Effects of BRP-187 on cell infiltration in zymosan-induced peritonitis. Male mice (n = 6, each group) were treated i.p. with BRP-187, MK886, zileuton or vehicle, 30 min before i.p. injection of zymosan. Analysis of leukocyte infiltration and MPO was performed 4 h after zymosan injection. $^{***}p < 0.001$ versus vehicle (*t*-test).

Treatment	Infiltration of leukocytes		MPO activity	
	$\times 10^6$	% inhibition	U/ml	% inhibition
Vehicle	7.6 ± 0.5	–	1.23 ± 0.13	–
10 mg/kg BRP-187	3.3 ± 0.6***	57%	0***	100%
1 mg/kg MK886	4.5 ± 0.3***	41%	0.72 ± 0.13***	41%

3 mg/kg) and it impaired leukocyte infiltration (41%) and MPO activity (42%) at 1 mg/kg (Tables 3 and 4).

4. Discussion

We here present the 4,5-diarylisoxazol-3-carboxylic acid derivative BRP-187 as highly potent inhibitor of cellular 5-LO product biosynthesis in human PMNL and monocytes with IC_{50} of 7–10 nM upon cell activation by LPS and fMLP that are pathophysiological relevant stimuli [34]. In mouse zymosan-induced peritonitis, BRP-187 exhibits marked effectiveness as LT biosynthesis inhibitor *in vivo*, and effectively suppressed vascular permeability and infiltration of leukocytes which are the major biological functions of cysLTs and LTB_4, respectively [2]. Our mechanistic studies using PLA suggest that BRP-187 acts primarily by preventing the 5-LO/FLAP complex assembly, a determinant for cellular LT biosynthesis [2,8], but affects 5-LO enzymatic activity only at micromolar concentrations, and fails to interfere with cellular AA release or cellular viability.

Our results suggest that BRP-187 apparently interacts with both, 5-LO and FLAP. Assignment of a given compound as FLAP inhibitor is hampered by the circumstance that (i) FLAP is operative only in intact cells and (ii) FLAP possesses no enzymatic activity or any function that can be easily monitored as read-out reflecting functional FLAP interference. Nevertheless, we provide several lines of evidence that BRP-187 interferes with the function of FLAP. First, our previous docking studies and MD simulations using the 3D structure of FLAP (PDB code 2Q7M) reveal concrete molecular interactions of BRP-187 with amino acids in the AA-binding pocket and thus, strongly support FLAP binding [21]. Second, the potent suppression of cellular 5-LO product biosynthesis in PMNL and monocytes was clearly impaired by provision of excess of exogenous AA, which is a typical feature for FLAP inhibitors [15,29,42]. Thus, FLAP facilitates access of endogenous AA to 5-LO [13,16,19] and supplementation of exogenous AA can circumvent the requirement for FLAP. Third, and strikingly, BRP-187 prevented the agonist-induced 5-LO/FLAP complex assembly in monocytes and PMNL analyzed by a previously established PLA [11], a method that allows studying *in-situ* analysis of intracellular protein-protein interactions [27]. This effect of BRP-187 was not due to general blockade of 5-LO translocation to the perinuclear region, even when higher concentrations of BRP187 (or MK886 as control) were used as compared to the PLA experiments. This is in contrast, to hyperforin that prevents membrane-binding of 5-LO via interaction with the C2-like domain [43]. Of note, MK886, a drug that was originally used as probe to identify FLAP [36], revealed the same pattern: preventing 5-LO/FLAP complex formation without blocking 5-LO translocation. In this respect, the direct 5-LO inhibitor zileuton (used as control) was not effective in either experiment, as expected and shown before [11].

Direct inhibition of 5-LO activity by BRP-187 is clearly evident in cell-free assays using PMNL homogenates and isolated human recombinant 5-LO as enzyme source. In such assays, pure FLAP inhibitors like MK886 are inactive [9,10,29,44]. Wash-out experiments and studies using the nonionic detergent Triton X-100 exclude irreversible 5-LO inhibition and unspecific (lipophilic) aggregate-induced 5-LO interference, respectively. Note that in contrast to direct redox-type and iron-chelating 5-LO inhibitors, the so-called "competitive nonredox-type" 5-LO inhibitors (such as CJ-13,610 [37] or ZM230487 [38]) show a similar pattern of differential efficiency in cell-free and cell-based 5-LO assays, that is, loss of potency in cell-free test systems. Therefore, BRP-187 could act as nonredox-type 5-LO inhibitor with high potency only in intact cells. However, lowering hydroperoxide levels by reconstitution of a reducing milieu (supplementation of GSH or DTT to PMNL homogenates) restores efficient 5-LO inhibition by nonredox-type inhibitors [45], which was not the case for BRP-187. Together, BRP-187 specifically and reversibly interferes with 5-LO, albeit only at 30- to 300-fold higher concentrations as compared to suppression of 5-LO product biosynthesis in intact PMNL or monocytes.

Because FLAP is member of the MAPEG family [46], we asked whether BRP-187 may affect also other enzymes out of this class, which in fact is the case for MK886 that blocks LTC_4 synthase [40] and mPGES-1 activity [39]. Although BRP-187 failed to inhibit AA release in intact cells (this study) or other AA-converting dioxygenases like COX-1/2 or 12/15-LOs [21], it markedly suppressed mPGES-1 activity and to a lower extent also LTC_4 synthase activity. In this respect, the high potency of BRP-187 against human mPGES-1 is remarkable (IC_{50} = 0.2 μM versus 2.4 μM for MK886 [41] in a similar assay) and might be of pharmacological relevance for the treatment of pain and inflammatory conditions. Further cellular and preclinical studies warrant evaluation of (human and rodent) mPGES-1 inhibition in more detail.

Despite the obvious value and benefit of anti-LT therapy for many LT-related diseases such as asthma and allergic rhinitis, CVD and cancer, there is still an unmet need for safe and efficient drugs suitable for intervention in pharmacotherapy [47]. In fact, there are currently several clinical trials ongoing with FLAP inhibitors and promising results encourage for preclinical evaluations of novel compounds [10]. Moreover, data from cohort studies suggest a link between CVD and FLAP [48–51] and increased the interest in developing agents that interfere with FLAP. It also seems that FLAP inhibitors might outperform direct 5-LO inhibitors in *in vivo* experiments, in preclinical studies, and in clinical trials [10]. In our LT-related model of murine peritonitis induced by zymosan [30], BRP-187 (as well as MK886) was highly efficient in reducing the cysLT levels *in vivo* after i.p. application and in this respect outperformed the direct 5-LO inhibitor zileuton. Accordingly, one of the major bioactivities of cysLTs, i.e. increase of vascular permeability [52], was significantly blocked under these conditions by BRP-187 but not so by zileuton at the same dose. Along these lines, infiltration of leukocytes into the peritoneal cavity, which is potently elicited by the powerful chemotactic LTB_4 [53], was inhibited by BRP-187 in the zymosan-induced peritonitis model. Therefore, our data support the concept of FLAP interference as pharmacological therapy of LT-related diseases and highlight BRP-187 as novel type of efficient inhibitor of the FLAP/5-LO complex assembly.

MK886, an indole derivate that inhibits LT formation *in vitro* and *in vivo* was used as tool/probe to identify FLAP [36], and thus

represented the first FLAP inhibitor [18,54] that was assessed up to phase II clinical studies. Subsequently, the quinoline-class compound BAY X1005 (syn. DG-031) from Bayer [55], and the quinoline-indole hybrid MK591 [56] were presented as FLAP inhibitors that effectively inhibited LT biosynthesis. Although these compounds could enter phase II clinical trials, the clinical evaluations were discontinued for unknown reasons. However, novel derivatives of MK886 such as AM103 and the follow-up compound AM803 (now GSK2190915) were recently developed that potently inhibited LTB_4 formation with acceptable pharmacokinetics and preclinical toxicology [44,57,58]. GSK2190915 underwent several phase II clinical trials for treatment of asthma with partially promising results.

Taken together, BRP-187 represents a novel chemotype of LT biosynthesis inhibitors with outstanding potency in human PMNL and monocytes activated under pathophysiological relevant conditions and with effectiveness *in vivo*. Using the PLA that visualizes the *in situ* interaction between 5-LO and FLAP in intact cells during LT formation, we demonstrate that BRP-187 prevents 5-LO/FLAP complex assembly without blocking 5-LO nuclear redistribution. In light of the unmet need for effective and safe anti-LT drugs and the novel therapeutic indication for FLAP inhibitors, BRP-187 deserves attention and further preclinical assessment of the compound is warranted.

Conflict of interest statement

None declared.

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