

RESEARCH ARTICLE

Leukotriene B₄ Metabolism and p70S6 Kinase 1 Inhibitors: PF-4708671 but Not LY2584702 Inhibits CYP4F3A and the ω-Oxidation of Leukotriene B₄ *In Vitro* and *In Cellulo*

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Abstract

LTB₄ is an inflammatory lipid mediator mainly biosynthesized by leukocytes. Since its implication in inflammatory diseases is well recognized, many tools to regulate its biosynthesis have been developed and showed promising results *in vitro* and *in vivo*, but mixed results in clinical trials. Recently, the mTOR pathway component p70S6 kinase 1 (p70S6K1) has been linked to LTC₄ synthase and the biosynthesis of cysteinyl-leukotrienes. In this respect, we investigated if p70S6K1 could also play a role in LTB₄ biosynthesis. We thus evaluated the impact of the p70S6K1 inhibitors PF-4708671 and LY2584702 on LTB₄ biosynthesis in human neutrophils. At a concentration of 10 μM, both compounds inhibited S6 phosphorylation, although neither one inhibited the thapsigargin-induced LTB₄ biosynthesis, as assessed by the sum of LTB₄, 20-OH-LTB₄, and 20-COOH-LTB₄. However, PF-4708671, but not LY2584702, inhibited the ω-oxidation of LTB₄ into 20-OH-LTB₄ by intact neutrophils and by recombinant CYP4F3A, leading to increased LTB₄ levels. This was true for both endogenously biosynthesized and exogenously added LTB₄. In contrast to that of 17-octadecynoic acid, the inhibitory effect of PF-4708671 was easily removed by washing the neutrophils, indicating that PF-4708671 was a reversible CYP4F3A inhibitor. At optimal concentration, PF-4708671 increased the half-life of LTB₄ in our neutrophil suspensions by 7.5 fold, compared to 5 fold for 17-octadecynoic acid. Finally, Michaelis-Menten and Lineweaver-Burk plots indicate that PF-4708671 is a mixed inhibitor of CYP4F3A. In conclusion, we show that PF-4708671 inhibits CYP4F3A and prevents the ω-oxidation of LTB₄ *in cellulo*, which might result in increased LTB₄ levels *in vivo*.

Introduction

Leukotrienes (LT) are inflammatory lipid mediators derived from arachidonic acid. They participate in the inflammatory cascade in numerous conditions, notably asthma, rheumatoid

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arthritis, allergies and in host defense [1, 2]. They are mainly biosynthesized by leukocytes via the 5-lipoxygenase (5-LO) pathway. With the help of its activating protein, 5-LO metabolizes arachidonic acid into the unstable intermediate LTA_4 . LTA_4 can subsequently be metabolized into LTB_4 by the LTA_4 hydrolase, or into LTC_4 by the LTC_4 synthase. Cysteinyl-LTs are well known for their role in asthma and bronchoconstriction, while LTB_4 is more involved in leukocyte recruitment and activation. In humans, LTB_4 can be further metabolized into 12-oxo- LTB_4 by the LTB_4 12-hydroxydehydrogenase, or it can be ω -oxidized by the CYP4F3A [3–5]. The latter, which is mainly expressed in neutrophils, catalyzes the formation of 20-OH- LTB_4 , then 20-COOH- LTB_4 .

The recognized implication of LTB_4 in inflammation makes it an attractive therapeutic target. However, the inhibition of LTB_4 biosynthesis showed mixed results in clinical trials, despite promising results in mice models of inflammatory diseases [1, 2]. Among the numerous compounds tested, only the 5-LO inhibitor Zileuton has been approved as a treatment for asthma. Therefore, a better understanding of LTB_4 metabolism and its regulation could lead to new therapeutic approaches.

Two recent studies linked the mTOR pathway component p70S6 kinase 1 (p70S6K1) to LTC_4 biosynthesis, showing that p70S6K1 could phosphorylate the LTC_4 synthase, hence modulating its activity [6, 7]. Herein, we sought to determine whether p70S6K1 could also modulate LTB_4 biosynthesis and metabolism. We thus evaluated the impact of two selective p70S6K1 inhibitors, PF-4708671 [8] and LY2584702 [9], on the biosynthesis of LTB_4 and its metabolites in human neutrophils.

Materials and Methods

Material

Lymphocyte separation medium, aprotinin, dimethyl sulfoxide (DMSO) and solvents for HPLC and LC/MS were purchased from Thermo Fisher Scientific (Ottawa, Ontario, Canada). Dextran, adenosine deaminase, leupeptin and potassium phosphate were obtained from Sigma-Aldrich Canada (Oakville, Ontario, Canada). HBSS was purchased from Wisent Bio-products (St-Bruno, Quebec, Canada). 19-OH-prostaglandin (PG) B_2 , PGB_2 , PGB_2 - D_4 and 17-octadecynoic acid (17-ODYA) were purchased from Cayman Chemicals (Ann Arbor, Michigan, USA). PF-4708671 was obtained from Abcam (Cambridge, Massachusetts, USA) and LY2584702 from Selleckchem (Houston, Texas, USA). Thapsigargin was obtained from Tocris Bioscience (Ellisville, Missouri, USA). LTB_4 was a generous gift from Dr Louis Flamand (Université Laval, Québec City, Canada). Recombinant CYP4F3A and the NADPH regenerating system were purchased from Corning (Corning, New York, USA). Protease and phosphatase inhibitor cocktail tablets were purchased from Roche (Laval, Quebec, Canada). Primary (anti-phospho-S6 #2211 and anti-S6 #2317) and secondary antibodies were obtained from Cell Signaling (Danvers, Massachusetts, USA). The enhanced chemiluminescent (ECL) substrate was obtained from Millipore Canada Ltd (Toronto, Ontario, Canada).

Preparation and utilization of adenosine deaminase

Adenosine deaminase was prepared and utilized exactly as described before [10].

Isolation of human neutrophils and cell stimulations

Human neutrophils were isolated from the peripheral blood of healthy volunteers, without consideration for gender, as described before [11]. For the experiments investigating the impact of p70S6 kinase inhibitors on LTB_4 biosynthesis and LTB_4 half-life, pre-warmed

human neutrophil suspensions (37°C, 5 million cells/ml in HBSS containing 1.6 mM CaCl₂) were incubated with PF-4708671, LY2584702 or vehicle (DMSO) for 5 minutes, then stimulated with 100 nM thapsigargin for 10 minutes or 1 μM LTB₄ for different times (see Figures). For experiments in which the reversibility of PF-4708671 and 17-ODYA were assessed, pre-warmed human neutrophil suspensions were incubated with PF-4708671, 17-ODYA or vehicle (DMSO) for 5, 15 or 30 minutes. Cells were centrifuged (350 × g) and the pellets were suspended in HBSS-CaCl₂ or autologous plasma for 20 minutes. Cells were washed 3 times with warm HBSS-CaCl₂ before adding 1 μM LTB₄ for 20 minutes.

Analysis of LTB₄ and its ω-oxidation products

Incubations were stopped by adding 1 volume of a cold (-30°C) stop solution (MeOH/MeCN, 1/1, v/v) containing 12.5 ng of both 19-OH-PGB₂ and PGB₂ as internal standards. The samples were placed at -30°C overnight to allow protein denaturation and then centrifuged (1000 × g, 10 minutes, 4°C). The resulting supernatants were analyzed by reversed-phase HPLC using a Shimadzu HPLC System (Shimadzu corporation, Kyoto, Japan) and an on-line extraction procedure based on a method from Borgeat *et al* [12]. In brief, samples were diluted 1/3 with water, then injected and loaded onto a C8 precolumn (Aquapore Octyl 7 μM, PerkinElmer, Waltham, USA) using water containing 0.01% phosphoric acid during 4.25 minutes. The C8 precolumn was then switched on-line with the HPLC analytical column (Accucore™ C18, 50 × 4.6 mm, 2.6 μm, ThermoFisher Scientific, Ottawa, Canada) and elution was performed using a discontinuous binary gradient with solvent A and solvent B. Solvent A consisted of MeOH/MeCN/H₂O at a 44/11/45 ratio (v/v/v) plus 0.01% AcOH and DMSO. Solvent B consisted of MeOH/MeCN/H₂O at a 63/32/5 ratio (v/v/v) plus 0.01% AcOH. The gradient was: 0–15% B from 4.25 to 5.25 minutes; 15–70% B from 5.25 to 12 minutes; 70–100% B from 12 to 12.30 minutes and held at 100% during 4 minutes. Column then was re-equilibrated into 15% solvent B during 5 minutes before the next sample was injected. Using this method, the retention times were 5.3 minutes for 19-OH-PGB₂, 6.4 minutes for 20-COOH-LTB₄, 6.7 minutes for 20-OH-LTB₄, 8.9 minutes for PGB₂, 9.8 minutes for 6Z-LTB₄, 9.9 minutes for 6Z-12epi-LTB₄, 10.1 minutes for LTB₄, and 12.5 minutes for 5-HETE. Internal standards and LTs were detected by UV at 270 nm while 5-HETE was detected at 235 nm. Leukotrienes represent the sum of LTB₄, 20-OH-LTB₄ and 20-COOH-LTB₄.

In vitro CYP4F3A assay

Human recombinant CYP4F3A (5 pg/ml) in potassium phosphate buffer (100 mM, pH 7.4) containing a NADPH generating system (glucose-6-phosphate, NADP⁺, glucose-6-phosphate dehydrogenase, MgCl₂) was warmed at 37°C then incubated for 5 minutes with inhibitors or vehicle (DMSO). LTB₄ (1–20 μM) then was added and reactions were stopped at different times with 5 volumes of a cold stop solution. LTB₄ and its ω-oxidation products were quantified by HPLC as described in methods. The initial reaction rate for each LTB₄ concentration was determined. The maximal velocity (v_{max}) and the Michaelis-Menten constant (K_M) were calculated for each concentration of PF-4708671 to assess the type of inhibition, using non-linear regression of the Michaelis-Menten graph with the Graphpad Prism 7 Software (GraphPad Software, Inc., La Jolla, California, USA). The Michaelis-Menten graph was also linearized using the Lineweaver-Burk (double reciprocal) plot.

Immunoblot

Pre-warmed neutrophil suspensions (37°C, 5 million cells/ml in HBSS containing 1.6 mM CaCl₂) were stimulated with 100 nM of thapsigargin or N-Formylmethionine-leucyl-

phenylalanine (fMLP) for 5 minutes. PF-4708671, LY2584702 or vehicle were added 5 minutes before stimulation. Incubations were stopped using 1 volume of cold (4°C) incubation buffer. The suspensions were centrifuged (350 x g, 5 min, 4°C) and then lysed in a cold (4°C) hypotonic buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM MgCl₂, 1 mM EDTA, pH 7.4) containing 0.1% NP-40, protease inhibitors (10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM PMSF, protease inhibitor cocktail tablets), 2 mM diisopropyl fluorophosphate (DFP) and phosSTOP. Cells were vortexed for 15 seconds, then immediately solubilized in electrophoresis sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 0.01% bromophenol blue, 5% β-mercaptoethanol, 2% SDS) and boiled for 10 minutes. Proteins were loaded on a 12% polyacrylamide gel for electrophoresis, and transferred onto a PVDF membrane. Membranes were blocked using TBS/Tween buffer containing 5% w/v skim milk and incubated overnight at 4°C with primary antibodies (anti-phospho-S6 #2211 and anti-S6 #2317, Cell Signaling) in TBS/Tween containing 5% skim milk. HRP-linked secondary antibodies and ECL substrate were used for detection.

Quantification of PF-4708671 by LC-MS/MS

Incubations were stopped by adding one volume of cold (-30°C) MeOH + 0.01% acetic acid containing 2 ng of PGB₂-D₄ as an internal standard. The samples were placed at -30°C overnight to allow protein denaturation and then centrifuged (1000 × g, 10 minutes). The resulting supernatants were collected and diluted with water + 0.01% acetic acid to obtain a final MeOH concentration ≤ 10%. Lipids were extracted from the samples using solid phase extraction cartridges (Strata-X Polymeric Reversed Phase, 60 mg/1ml, Phenomenex). The eluate was dried under a stream of nitrogen and reconstituted in 50 µl of MeOH. 1 µl was injected onto an HPLC column (Kinetex C8, 150 × 2.1 mm, 2.6 µm, Phenomenex) and eluted at a flow rate of 500 µl/min with a linear gradient using 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). The gradient lasted 20 minutes, starting at 10:90 (A:B) a finishing at 90:10 (A:B). The HPLC system was interfaced with the electrospray source of a Shimadzu 8050 triple quadrupole mass spectrometer and mass spectrometric analysis was done in the negative ion mode using multiple reaction monitoring for the specific mass transition m/z 389.10 → 197.95.

Statistical analyses

Data are represented as the mean ± S.D. All calculations were done using the Graphpad Prism 7 Software.

Ethics

This study was approved by the local ethics committee (Comité d'éthique de la recherche de l'Institut universitaire de cardiologie et de pneumologie de Québec) and all subjects signed a consent form.

Results

p70S6 kinase 1 inhibitors do not inhibit the biosynthesis of LTB₄ but PF-4708671 prevents further LTB₄ metabolism

In the first series of experiments, we investigated whether p70S6K1 inhibitors modulated LTB₄ biosynthesis. As shown in Fig 1A, PF-4708671 and LY2584702 at a concentration of 10 µM did not stimulate nor inhibit the thapsigargin-induced LTB₄ biosynthesis. At this concentration, both compounds inhibited the phosphorylation of S6 induced by thapsigargin or fMLP (Fig 1B), indicating that they were both efficiently inhibiting the p70S6K1. However, we

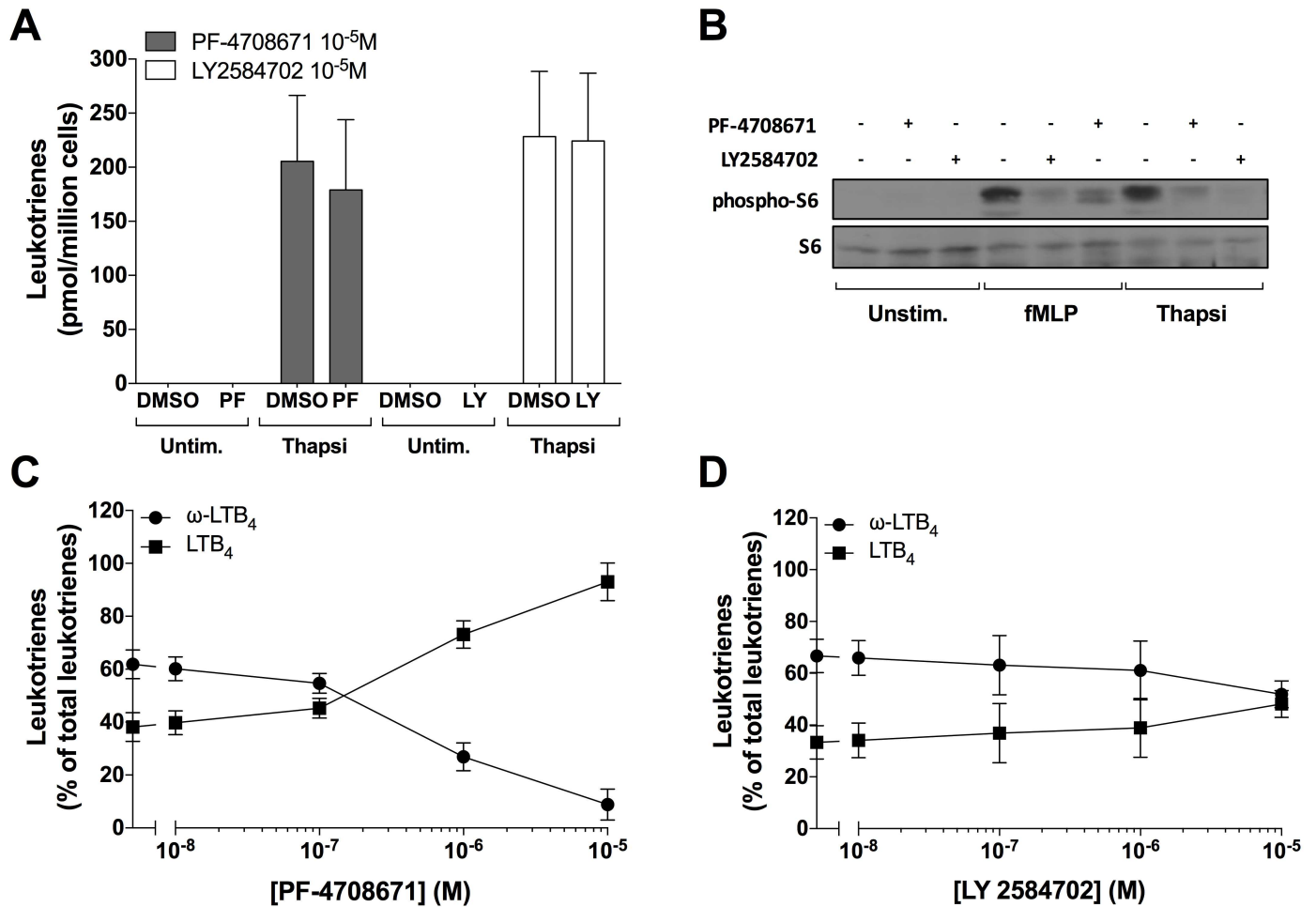


Fig 1. Impact of the p70S6K1 inhibitors on LTB₄ biosynthesis and ω-oxidation in neutrophil suspensions. A-D) Pre-warmed human neutrophil suspensions (37°C, 5 million cells/ml in HBSS containing 1.6 mM CaCl₂) were incubated with PF-4708671, LY2584702 or vehicle (DMSO) for 5 minutes, then stimulated with 100 nM thapsigargin for 10 minutes. A,C,D) Samples were processed and analyzed for LTB₄ biosynthesis as described in methods. Data are the mean (± S.D) of 5 independent experiments, each performed in duplicate. A) Leukotrienes represent the sum of LTB₄, 20-OH-LTB₄ and 20-COOH-LTB₄. C,D) ω-LTB₄ represents the sum of 20-OH-LTB₄ and 20-COOH-LTB₄. B) Samples were processed and analyzed for S6 and phospho-S6 content as described in methods. Data are from one experiment, representative of three.

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noticed that 10 μM PF-4708671 prevented the metabolism of LTB₄ into 20-OH-LTB₄ and 20-COOH-LTB₄ in thapsigargin-stimulated neutrophils with an IC₅₀ of ~800 nM (Fig 1C). This led to a decrease in 20-OH- and 20-COOH-LTB₄ levels, and an increase in LTB₄ levels. In contrast, LY2584702 did not prevent the metabolism of LTB₄ into 20-OH- and 20-COOH-LTB₄ in thapsigargin-stimulated neutrophils (Fig 1D).

We next determined if PF-4708671 could also inhibit the degradation of exogenously added LTB₄ to 20-OH- and 20-COOH-LTB₄ by performing kinetic experiments in which neutrophils were incubated with 1 μM LTB₄. PF-4708671 increased the half-life of LTB₄ by 7.5 fold, from ~20 minutes to ~150 minutes (Fig 2A). In contrast, LY2584702 did not significantly modulate LTB₄ half-life (Fig 2B). In comparison, the CYP4F3A inhibitor 17-ODYA, previously shown to inhibit the metabolism of LTB₄ into 20-OH-LTB₄ in human neutrophils [13], increased LTB₄ half-life by 5 fold, from ~10 minutes to ~50 minutes (Fig 2C). In these experiments, neutrophils were treated with PF-4708671 and 17-ODYA during 5 and 30 minutes

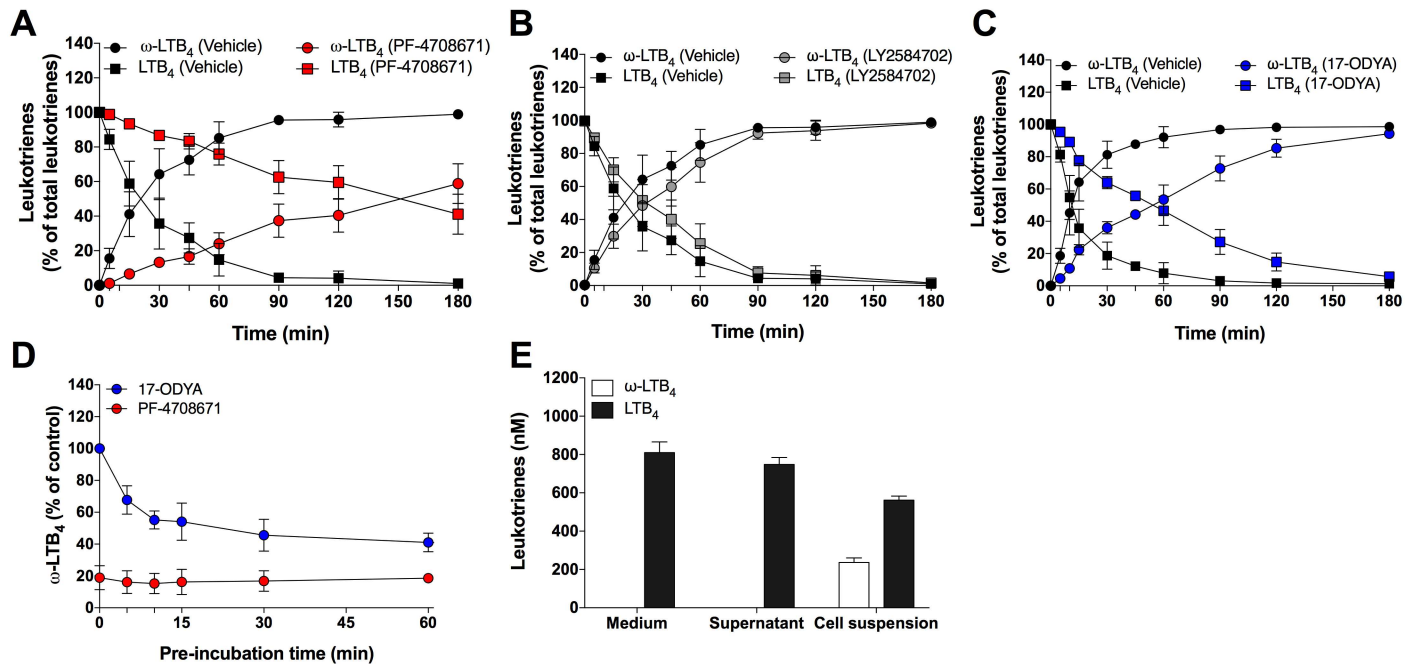


Fig 2. Impact of p70S6K1 and CYP4F3A on the half-life of LTB₄ in cellulo. Pre-warmed human neutrophil suspensions (37°C, 5 million cells/ml in HBSS containing 1.6 mM CaCl₂) were incubated 5 minutes with vehicle (DMSO), **A**) PF-4708671 (30 μM), **B**) LY2584702 (10 μM), or 30 minutes with **C**) 17-ODYA (30 μM), then treated with 1 μM of LTB₄ for the indicated times. **D**) PF-4708671 (30 μM) or 17-ODYA (30 μM) were added to neutrophil suspensions for the indicated times before adding 1 μM LTB₄ for 20 minutes. **E**) LTB₄ (1 μM) was added for 20 minutes to either incubation medium, neutrophil supernatants (incubation medium incubated with neutrophils during 30 minutes) or neutrophil suspensions. **A–E**) Samples then were processed and analyzed for LTB₄ biosynthesis as described in methods. ω-LTB₄ represents the sum of 20-OH-LTB₄ and 20-COOH-LTB₄. Data are the mean (± S.D.) of 4–5 independent experiments, each performed in duplicate.

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respectively before the addition of LTB₄. This is because PF-4708671 exerted its inhibitory constraint almost instantaneously while the optimal inhibitory effect of 17-ODYA was observed after 30 minutes (Fig 2D). Of note, the conversion of LTB₄ into 20-OH- and 20-COOH-LTB₄ only occurred when neutrophils were present in the incubation media (Fig 2E). Indeed, LTB₄ was not transformed into ω-LTB₄ in our incubation medium (HBSS) or in a neutrophil supernatant, but was efficiently ω-oxidized in neutrophil suspensions, supporting the fact that the ω-oxidation of LTB₄ is an intracellular event.

PF-4708671 is a mixed inhibitor of CYP4F3A

The distinct inhibitory profiles of PF-4708671 and LY2584702 on LTB₄ metabolism raised the possibility that PF-4708671 was a CYP4F3A inhibitor. We thus tested this hypothesis by comparing the effect of p70S6K1 inhibitors with the CYP4F3A inhibitor 17-ODYA on human recombinant CYP4F3A activity. Human recombinant CYP4F3A was incubated with increasing concentrations of PF-4708671 or 17-ODYA for 5 and 30 minutes respectively, before the addition of 1 μM of LTB₄ for 1 minute. PF-4708671 induced a concentration-dependent inhibition of ω-oxidation product formation (IC₅₀ ~750 nM) while LY2584702 poorly affected this enzymatic conversion (Fig 3A). In other experiments, CYP4F3A was treated with increasing concentrations of PF-4708671 before the addition of various LTB₄ concentrations. We then calculated the maximal rate of the reaction (v_{max}) and the Michaelis-Menten constant (K_M) during the steady-state of the reaction using the non-linear regression of the Michaelis-Menten graph. The representation of the data using either the Michaelis-Menten graph (Fig 3C) or the

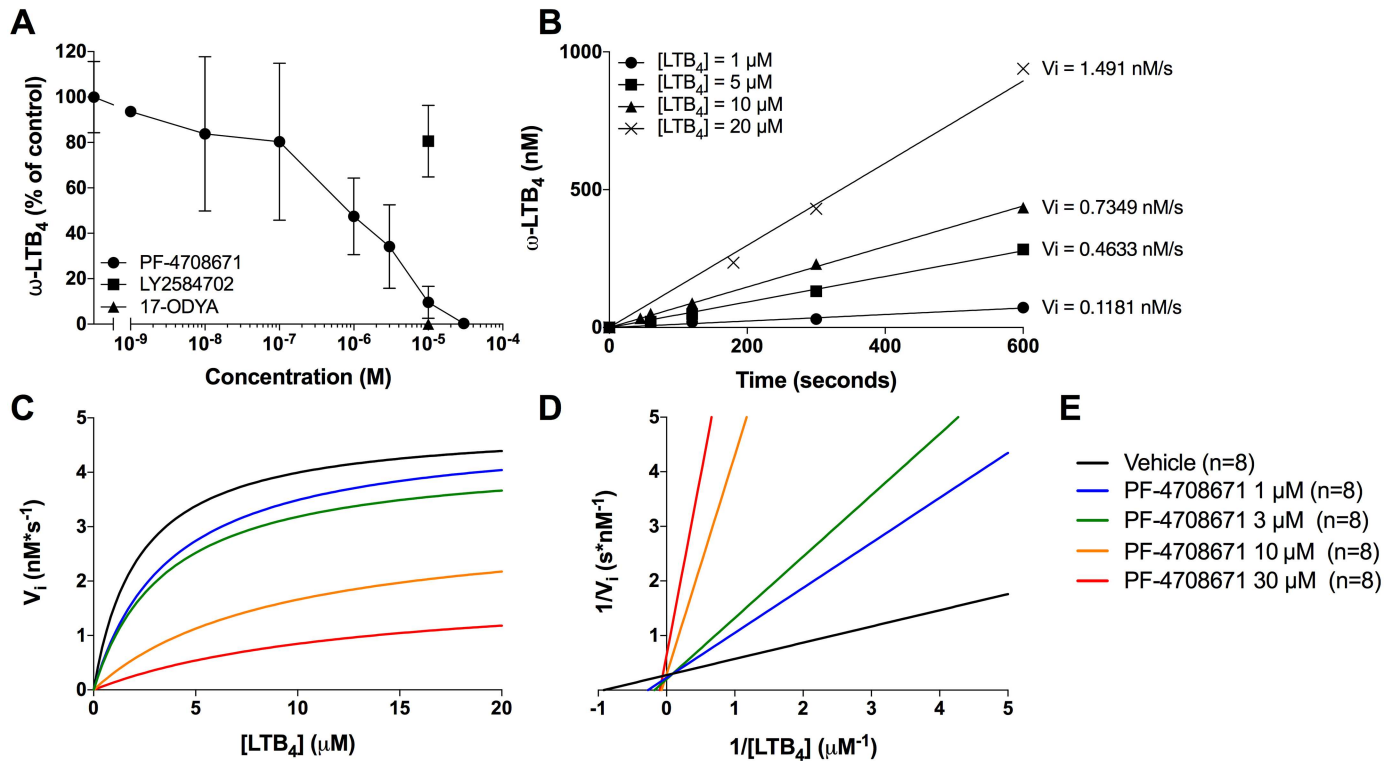


Fig 3. Impact of PF-4708671 on human recombinant CYP4F3A activity. **A)** Pre-warmed human recombinant CYP4F3A in phosphate buffer containing a NADPH regenerating system was incubated with increasing concentrations of PF-4708671 for 5 minutes, with LY2584702 (10^{-5} M) for 5 minutes or with 17-ODYA (10^{-5} M) for 30 minutes before the addition of $1 \mu\text{M}$ LTB₄ for 1 minute. **B)** Representative time curves of the ω -oxidation of LTB₄ par CYP4F3A. Initial rates were calculated by linear regression. **C)** Michaelis-Menten kinetics of the inhibition of CYP4F3A by PF-4708671. Human recombinant CYP4F3A was incubated with increasing concentrations of PF-4708671 before the addition of LTB₄ at different concentrations. LTB₄, and ω -LTB₄ (20-OH-LTB₄ and 20-COOH-LTB₄) were quantified by HPLC as described in methods and represent the addition of 20-OH-LTB₄ and 20-COOH-LTB₄. Data are shown as the mean (\pm S.D.) of 8 independent experiments. **D)** Data from the Michaelis-Menten graph were transformed using the Lineweaver-Burk plot (double reciprocal plot). **E)** Legend for C and D.

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Lineweaver-Burk plot (Fig 3D) are consistent with a model of mixed inhibition. This mixed inhibition indicated that PF-4708671 was perhaps a substrate of CYP4F3A. However, LC-MS/MS analyses of PF-4708671 levels indicated that the compound remained stable in our neutrophil suspensions and in our enzymatic assay with recombinant CYP4F3A for up to 2 hours (data not shown).

The inhibitory effect of PF-4708671 on LTB₄ ω -oxidation is reversible

Finally, we assessed if the inhibitory effect of PF-4708671 was reversible by comparing it with the irreversible CYP4F3A inhibitor 17-ODYA [13]. In these experiments, neutrophils were incubated with PF-4708671 for 5 minutes or 17-ODYA for 30 minutes (optimal incubation times) or with the inhibitors for 15 minutes (comparable incubation time). Then, neutrophils were either washed with HBSS-CaCl₂, washed with autologous plasma or not washed at all, before treatment with either 100 nM thapsigargin or $1 \mu\text{M}$ LTB₄. In absence of washing, both PF-4708671 and 17-ODYA inhibited the metabolism of LTB₄ into 20-OH- and 20-COOH-LTB₄ (Fig 4). Washing the PF-4708671-treated neutrophils with either HBSS-CaCl₂ or autologous plasma restored the ability of neutrophils to metabolize endogenously formed and exogenously added LTB₄ into 20-OH- and 20-COOH-LTB₄ (Fig 4A–4C). In contrast, 17-ODYA-treated neutrophils that were washed with either HBSS-CaCl₂ or

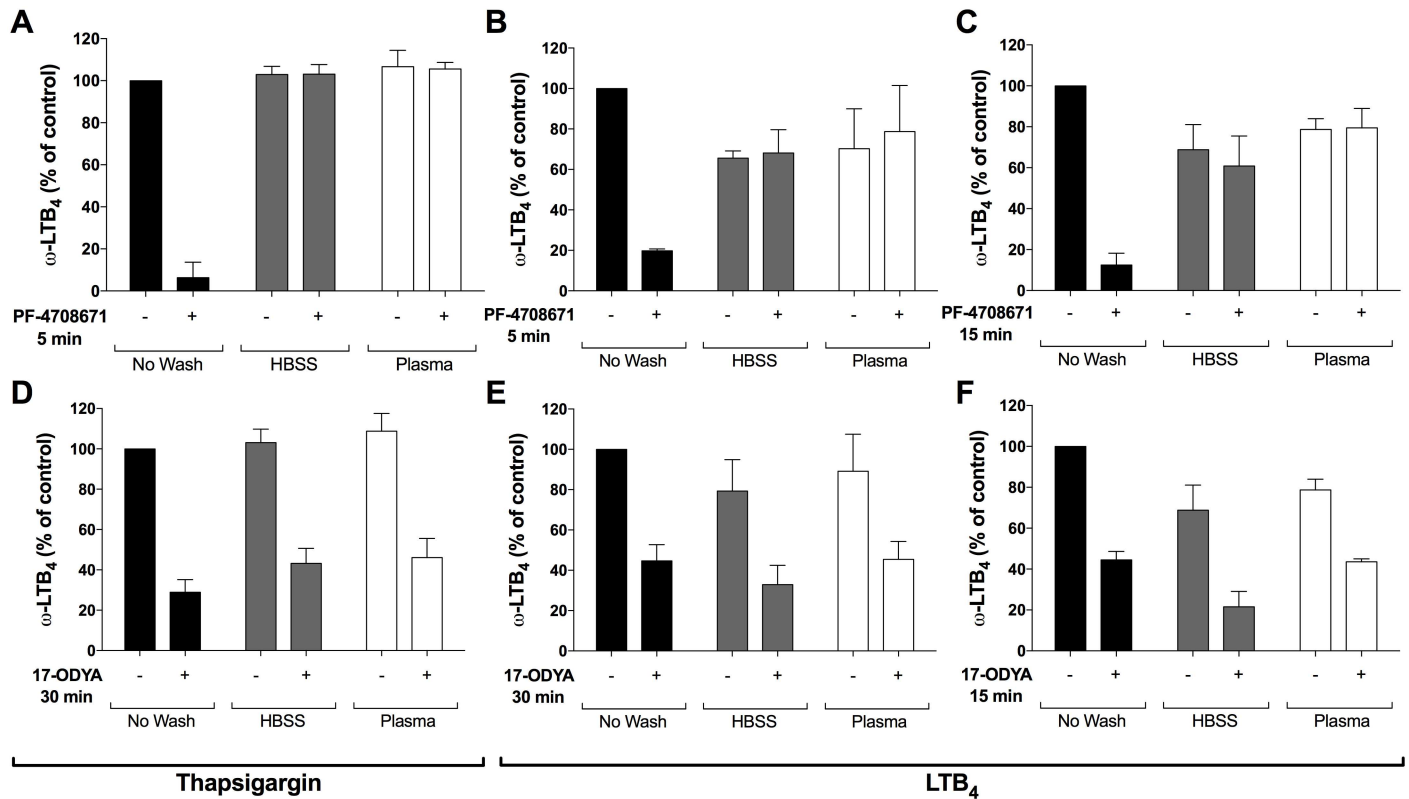


Fig 4. Removal of the inhibitory constraint exerted by CYP4F3A inhibitors on LTB₄ ω -oxidation in neutrophils. Pre-warmed human neutrophil suspensions (37°C, 5 million cells/ml in HBSS containing 1.6 mM CaCl₂) were incubated with **A,B**) 30 μ M PF-4708671 or vehicle for 5 minutes, **C**) 30 μ M PF-4708671 for 15 minutes, **D,E**) 30 μ M 17-ODYA for 30 minutes, or **F**) 30 μ M 17-ODYA for 15 minutes. Neutrophils were washed (or not) with autologous plasma or HBSS-CaCl₂ as described in methods. **A,D**) 100 nM thapsigargin or **B,C,E,F**) 1 μ M LTB₄ were then added for 10 and 20 minutes, respectively. Samples then were processed and analyzed for 20-OH-LTB₄ and 20-COOH-LTB₄ as described in methods. Data are the mean (\pm S.D.) of 4 independent experiments, each performed in duplicate.

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autologous plasma remained incapable of metabolizing endogenously biosynthesized or exogenously added LTB₄ into 20-OH- or 20-COOH-LTB₄ (Fig 4D–4F). Furthermore, similar results were obtained whether we used the optimal incubation time for each inhibitor or the comparable incubation time (5 and 30 minutes for PF-4708671 and 17-ODYA vs. 15 minutes for each inhibitor). These experiments indicate that in contrast to the irreversible inhibitor 17-ODYA, the inhibitory constraint of PF-4708671 is easily removable and that the latter is a reversible inhibitor of the CYP4F3A enzyme.

Discussion

Two recent studies have linked the mTOR pathway component p70S6K1 to LTC₄ synthase function, providing a possible new way of regulating cysteinyl-LT biosynthesis [6, 7]. Thus, we wondered if p70S6K1 could play a similar role in the regulation of LTB₄ synthesis. In that regard, our data show that 1) the thapsigargin-induced LTB₄ biosynthesis is not inhibited by p70S6K1 inhibitors; 2) PF-4708671, but not LY2584702, inhibits the metabolism of LTB₄ into 20-OH- and 20-COOH-LTB₄ in a concentration-dependant manner; 3) PF-4708671, but not LY2584702, inhibits human recombinant CYP4F3A; 4) PF-4708671 is a reversible inhibitor of CYP4F3A; and 5) PF-4708671 is a mixed inhibitor of CYP4F3A.

In this study, we aimed at documenting the inhibitory effect of p70S6K1 inhibitors on the ability of human neutrophils to metabolize LTB₄ into 20-OH- and 20-COOH-LTB₄. For that reason, we utilized an experimental model in which the priming of the arachidonic acid cascade and the 5-LO pathway were not involved, i.e. thapsigargin-stimulated neutrophils. In that experimental model, we could show that the ability of neutrophils to biosynthesize LTB₄, which includes the sum of LTB₄, 20-OH-LTB₄, and 20-COOH-LTB₄, was unchanged. This indicates that at the concentration utilized, the p70S6K1 inhibitors PF-4708671 and LY2584702 did not inhibit the enzymes involved in LTB₄ biosynthesis in human neutrophils, notably cPLA₂α, 5-LO, 5-LO-activating protein and LTA₄ hydrolase [14–17]. However, it remains possible that the p70S6K1 inhibitors used in this study might impact LTB₄ biosynthesis in human neutrophils when other signaling mechanisms are involved, notably those linked to the priming of LTB₄ biosynthesis such as cytokines and TLR activation [18, 19], raising the possibility that PF-4708671, and possibly LY2584702, modulate LT biosynthetic pathways through multiple mechanisms of action.

We found that PF-4708671, but not LY2584702, inhibited the metabolism of LTB₄ into 20-OH- and 20-COOH-LTB₄ in a concentration-dependant manner. In that regard, PF-4708671 was more potent than 17-ODYA, a recognized CYP4F3A inhibitor [13]. Moreover, the effect of PF-4708671 was more pronounced than that of 17-ODYA. While PF-4708671 increased the half-life of LTB₄ in our human neutrophil suspensions by 7.5 fold, 17-ODYA increased it by 5 fold. This indicates that PF-4708671 might be a promising tool to develop specific and potent inhibitors of the CYP4F3A enzyme.

Given that the Michaelis-Menten and the Lineweaver-Burk plots indicated that PF-4708671 was a mixed inhibitor, we thought that perhaps PF-4708671 was a CYP4F3A substrate and was metabolized to some extent by the enzyme. However, this hypothesis was proven incorrect, as PF-4708671 was stable for at least 2 hours in the presence of either recombinant CYP4F3A or human neutrophils. The mixed inhibition we observed also raised the possibility that a contaminant in our commercial PF-4708671 preparation might also inhibit CYP4F3A. Although we cannot confirm that possibility, we tested three different batches of PF-4708671 which all yielded the same results, indicating that the effects we are documenting are very unlikely to be caused by a compound that is present in trace amounts.

This is not the first study to underscore a non-specific effect of PF-4708671. Another group reported an off-target effect of PF-4708671 in immortalized mouse fibroblasts, showing that PF-4708671 activates AMPK and inhibits the mitochondrial respiratory chain complex I, independently of p70S6K1 [20]. Moreover, PF-4708671 is used as a tool to study the mTOR pathway in various *in vivo* and *in vitro* studies, mainly in models of type 2 diabetes and cancer [21–33]. In light of our findings, it cannot be ruled out that some of the previously reported effects of PF-4708671 are caused by its lack of specificity and possibly increased LTB₄ levels.

It was previously reported that LTB₄ levels are increased in white adipose tissue, liver and muscles of mice fed with a high fat diet [34, 35]. Furthermore, mice lacking LTB₄ receptor 1 are less susceptible to diet-induced insulin resistance [36, 37]. Therefore, using PF-4708671 in models of type 2 diabetes could increase LTB₄ levels and possibly lead to an increased inflammation. However, in rodents, the functional orthologue of CYP4F3A is CYP4F18, which transforms LTB₄ into 18-OH-LTB₄ instead of 20-OH-LTB₄ [38, 39]. While the activity of CYP4F18 towards LTB₄ has been characterized, it is still unknown whether PF-4708671 exerts an inhibitory effect on CYP4F18 as well.

In conclusion, we demonstrate that PF-4708671 is a reversible CYP4F3A inhibitor preventing the metabolism of LTB₄ into 20-OH- and 20-COOH-LTB₄. In addition to characterizing a new compound that induces a sustained elevation in LTB₄ levels, our data shed some light on the non-specific effects of a widely used p70S6K1 inhibitor. Given that it is more potent than

the only currently available CYP4F3A inhibitor, PF-4708671 might be an helpful tool for the development of potent CYP4F3A inhibitors to study the regulation of LTB₄ metabolism and its impact in inflammation.

Supporting Information

S1 Appendix.
(XLSX)

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