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A sensitive fluorogenic substrate for selective in vitro and in vivo assay of leukotriene A4 hydrolase activity



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ABSTRACT

Leukotriene A4 hydrolase (LTA4H) is a bifunctional zinc-dependent metalloprotease bearing both an epoxide hydrolase, producing the pro-inflammatory LTB4 leukotriene, and an aminopeptidase activity, whose physiological relevance has long been ignored. Distinct substrates are commonly used for each activity, although none is completely satisfactory; LTA4, substrate for the hydrolase activity, is unstable and inactivates the enzyme, whereas aminoacids β -naphthylamide and *para*-nitroanilide, used as aminopeptidase substrates, are poor and nonselective. Based on the three-dimensional structure of LTA4H, we describe a new, specific, and high-affinity fluorogenic substrate, **PL553** [*L*-(4-benzoyl)phenylalanyl- β -naphthylamide], with both in vitro and in vivo applications. **PL553** possesses a catalytic efficiency (k_{cat}/K_m) of $3.8 \pm 0.5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ using human recombinant LTA4H and a limit of detection and quantification of less than 1 to 2 ng. The **PL553** assay was validated by measuring the inhibitory potency of known LTA4H inhibitors and used to characterize new specific amino-phosphinic inhibitors. The LTA4H inhibition measured with **PL553** in mouse tissues, after intravenous administration of inhibitors, was also correlated with a reduction in LTB4 levels. This authenticates the assay as the first allowing the easy measurement of endogenous LTA4H activity and in vitro specific screening of new LTA4H inhibitors.

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Leukotriene A4 hydrolase (LTA4H,¹ EC 3.3.2.6) is a monomeric, bifunctional enzyme of 610 amino acids expressed in a wide variety of species—from bacteria to human—and ubiquitously distributed [1]. It is particularly abundant in intestine, spleen, lung (epithelial cells and alveolar macrophages), kidney, and placenta [1]. In blood, neutrophils, monocytes, lymphocytes, and erythrocytes are rich sources of this enzyme. LTA4H was originally identified as a member of the “arachidonic cascade,” where it catalyzes the hydrolysis of the unstable epoxide LTA4 into the potent pro-inflammatory diol LTB4 [2]. LTB4 has been involved in inflammatory diseases such as asthma, inflammatory bowel disease, chronic obstructive pulmonary disease, arthritis, psoriasis, atherosclerosis, and inflammatory pain

[3,4], in certain types of cancers [5], and in immunoregulatory processes (by stimulating the production of cytokines [6]). Therefore, potent inhibitors of LTA4H decreasing LTB4 biosynthesis could have wide therapeutic applications, and the enzyme is a target for drug discovery [3–5].

LTA4H has been purified from several mammalian sources (for reviews, see Ref. [7]) as well as cloned and sequenced from human lung and placenta [8]. The mouse [9], rat [10], and guinea pig [11] orthologs have also been isolated, and alignment of their sequences shows high protein conservation. The primary sequence analysis of this enzyme and its ability to cleave classical chromogenic aminopeptidase substrates in vitro first unraveled the aminopeptidase nature of LTA4H [12–14]. Site-directed mutagenesis experiments thereafter demonstrated that LTA4H belongs to the M1 family of the MA clan of zinc-dependent metalloproteases. Recent work has shown Pro-Gly-Pro, a neutrophil chemoattractant peptide, to be a physiological substrate of LTA4H, releasing the N-terminal proline residue [15]. These findings bring to light potential novel physiological functions for this enzyme beyond its LTA4 epoxide hydrolase activity.

Crystallographic studies [16] and site-directed mutagenesis experiments allowed the delineation of the amino acids specifically involved in either the epoxide hydrolase or the aminopeptidase activity, evidencing an overlap of the catalytic sites [17]. Thus, the catalytic Zn^{2+} ion and amino acids Glu271, Glu296, and

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¹ Abbreviations used: LTA4H, leukotriene A4 hydrolase; HPLC, high-performance liquid chromatography; APN, aminopeptidase N; APB, aminopeptidase B; 3D, three-dimensional; TBTU, 2-((1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro phosphate); β -NA, β -naphthylamine; DIEA, *N,N*-diisopropylethylamine; TFA, trifluoroacetic acid; UV-Vis, ultraviolet-visible; NMR, nuclear magnetic resonance; ME, methyl ester; APA, aminopeptidase A; LAP, leucine aminopeptidase; ERAP-1, endoplasmic reticulum aminopeptidase-1; ERAP-2, endoplasmic reticulum aminopeptidase-2; APP, aminopeptidase P; NEP, neutral endopeptidase; ACE, angiotensin-converting enzyme; FAAH, fatty acid amide hydrolase; EDTA, ethylenediaminetetraacetic acid; LOD, limit of detection; LOQ, limit of quantitation; AMC, 7-amido-4-methylcoumarin; PMSF, phenylmethanesulfonyl fluoride; RFU, relative fluorescent units.

Tyr383 are essential for the aminopeptidase activity, whereas Zn²⁺, Glu271, Arg563, and Asp375 are essential, and Glu296 is important, for the epoxide hydrolase activity. Due to this structural overlap, inhibitors of LTA4H generally act on both activities.

The epoxide hydrolase activity of LTA4H can be monitored by the hydrolysis of the natural LTA4 substrate into LTB4 by high-performance liquid chromatography (HPLC) or enzyme-linked immunosorbent assay (ELISA) [18]. These tests can be performed using either purified enzyme or whole blood as a source of both endogenous LTA4 substrate and LTA4H. Recently, a miniaturization of this assay using the homogeneous time-resolved fluorescence (HTRF) technology was described [19]. However, the chemical instability of LTA4 and the suicide inactivation of the enzyme by its natural substrate make it difficult to use this assay for inhibitor screening. As a result, the aminopeptidase assay of the enzyme is often used to assess the inhibitory potencies of molecules. Aminopeptidase assays of LTA4H measure chromogenic or fluorogenic substrate hydrolysis, such as Ala-, Leu-, Lys-, and Arg-*para*-nitroanilide or - β -naphthylamide [12–14], eventually associated with a competing tripeptide substrate [20]. However, because these substrates are also cleaved by other widely expressed aminopeptidases such as aminopeptidase N (APN) and aminopeptidase B (APB), these assays can only be used in vitro with pure enzyme. Consequently, two tests are generally performed for the design and/or screening of potential inhibitors: a rapid in vitro, nonspecific, aminopeptidase assay and a more complex in vitro hydrolase assay with exogenous LTA4. These two tests are generally associated with the whole blood assay that reflects the in vivo activity of drugs.

The aim of this work was to simplify these methods by designing a novel specific peptide substrate of LTA4H that could be used for both in vitro and in or ex vivo activity measurement. For this purpose, the model for LTA4 interaction within the active site of the enzyme proposed by Thunnissen and coworkers [21] and based on the three-dimensional (3D) structure of LTA4H–inhibitor complexes was used [16,22]. This model suggested a large binding site with the Arg563 and Lys565 amino acids forming the carboxylate recognition site. The S₁ subsite of the enzyme active site, as defined in the nomenclature of Schechter and Berger [23], was shown to form a deep pocket essentially lined by hydrophobic amino acids, whereas the catalytic zinc was in a suitable position for the hydrolysis of either the allylic epoxide of LTA4 or the amide bond of a peptide substrate.

Starting from these data, we designed new fluorogenic β -naphthylamide substrates containing extended side chains expected to fit the S₁ subsite precluding recognition by other metallopeptidases, particularly APN. Accordingly, one of these compounds, L-(4-benzoyl)phenylalanine- β -naphthylamide (further designated as **PL553**), was efficiently and selectively cleaved by LTA4H, as compared with a panel of related metalloproteases. To validate the use of this novel substrate, the inhibitory potencies of various LTA4H inhibitors were measured and compared with those obtained using L-Ala- β -naphthylamine. Finally, the **PL553** assay was used to assess the ex vivo activity of LTA4H in different tissues of mice treated with known and novel inhibitors. The measurement of the reduction in LTB4 levels in the same tissue demonstrated the interest of **PL553** assay to convey inhibitor potency and pharmacological effects.

Materials and methods

Reagents

The N-protected amino acids, Boc-(L)-(4-nitro)-Phe-OH, Boc-(L)-Bip-OH, Boc-(L)-Bpa-OH, Boc-(L)-Tyr(Bzl)-OH, and Fmoc-(L)-Lys

(Dnp)OH, were purchased from Bachem (Weil am Rhein, Germany). The coupling reagent TBTU [2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro phosphate] was purchased from Polypeptides (Strasbourg, France). All other reagents were obtained from Sigma–Aldrich, and the solvents were obtained from Carlo Erba–SDS (France). The (L)-Ala- β -naphthylamide substrate was purchased from Sigma. Purified human recombinant LTA4H was a generous gift from J.Z. Haeggström. APN from porcine kidney was purchased from Sigma. LTB4 concentrations were measured using the enzyme immunoassay from Sapphire Bioscience (USA).

Peptide synthesis

The fluorogenic β -naphthylamides **1a** to **1f** were synthesized by coupling β -naphthylamine (β -NA) at the C terminus of the different Boc and Fmoc amino acids using TBTU and DIEA (*N,N*-diisopropylethylamine). Cleavage of the Boc group was obtained by treatment with trifluoroacetic acid (TFA) in CH₂Cl₂, and cleavage of the Fmoc group was obtained by using 20% piperidine in dimethylformamide. The final products were purified by semipreparative HPLC. The synthetic pathway used for the preparation of the various substrates is summarized in [Supplemental Fig. 1 of the supplementary material](#).

Peptide purity was verified by HPLC using a reverse phase Kromasil C18 column (250 \times 4.6 mm, 5 μ m, 100 Å) with H₂O (0.1% TFA) (solvent A)/CH₃CN (0.1% TFA) (solvent B) as mobile phase using isocratic conditions, at a flow rate of 1 ml/min, on a Prominence Shimadzu LC20AB apparatus equipped with an ultraviolet (UV) detector. Elution of the peaks was monitored at 210 nm. Peptide structures were confirmed by ¹H nuclear magnetic resonance (NMR) spectroscopy (Bruker, 200 MHz) in dimethyl sulfoxide (DMSO)-d₆ or CDCl₃. Mass spectra were determined using an Agilent ion trap electrospray in positive mode mass spectrometer. For further details on synthesis, see [Supplemental Data “Experimental Procedures” in supplementary material](#).

Substrate docking

The 3D coordinates of the substrates were generated with Discovery Studio version 3.5. Flexible docking of the substrates within the binding subsites constituting the active site of LTA4H was performed using GOLD 5.1.

Synthesis of LTA4H inhibitors

Compound **2** and its methyl ester (ME) were synthesized as described in the literature [24]. Phosphinic inhibitors **3**, **4**, and **5** and their MEs were synthesized in eight steps starting from commercially available carboxylic acids. All compounds were purified by semipreparative reverse phase HPLC, and their purity, assessed by analytical HPLC, electrospray ionization (ESI) (+), and NMR analysis, showed that they were all more than 95% pure. For further details on synthesis, see [Supplemental Data “Experimental Procedures”](#).

UV and fluorimetric spectra

UV absorption spectra of a 10- μ M solution of compound and of its metabolite, β -NA, in 1 ml of 50 mM Tris–HCl (pH 7.4) and 100 mM NaCl were recorded using a UV–Vis (visible) spectrometer Shimadzu UV–Vis 1240. Spectrofluorimetric emission spectra of the same solutions, diluted in 1 ml of 50 mM Tris–HCl (pH 7.4) and 100 mM NaCl, were recorded using a PerkinElmer LS 50B fluorimeter (λ_{ex} = 340 nm) equipped with a thermostatic cell holder.

LTA4H versus APN enzymatic activities using synthesized substrates

Initial screening of the synthesized β -naphthylamide peptide substrates was performed by comparing their produced fluorescent signal in the presence of either recombinant human LTA4H (1.2 $\mu\text{g}/\text{ml}$) in 50 mM Tris–HCl (pH 7.4) and 100 mM NaCl or with APN purified from porcine kidney (0.33 mU/ml) in 50 mM Tris–HCl (pH 7.4). Each substrate was tested at 20 μM , and the reactions were incubated in a final volume of 100 μl at 37 °C for 60 min. The fluorescent signals were read on a Twinkle LB 970 microplate fluorimeter (Berthold, λ_{ex} = 340 nm, λ_{em} = 405 nm, lamp energy = 10,000).

pH optimum of PL553 assay and salt addition

Recombinant human LTA4H (1.2 $\mu\text{g}/\text{ml}$) was used with a fixed concentration of PL553 (40 μM) in 100 μl of either 50 mM Mes buffer (pH 5.0 or 6.5) or 50 mM Tris (pH 6.5–9.0). The same experiments were also performed with the addition of 100 mM NaCl to the buffer. For each condition tested, the fluorescence of the substrate alone was also measured and subtracted from the fluorescent signal observed in the presence of LTA4H. The reactions were left to proceed for 60 min at 37 °C, and the fluorescent signal was read on a Twinkle LB 970 fluorimeter (Berthold, λ_{ex} = 340 nm, λ_{em} = 405 nm, lamp energy = 10,000).

Kinetic parameters of PL553 toward LTA4H

Determination of the K_m was performed in initial rate conditions. For this purpose, recombinant human LTA4H (0.6 $\mu\text{g}/\text{ml}$) was added to increasing concentrations of PL553 in a final volume of 100 μl of 50 mM Tris–HCl (pH 7.4) and 100 mM NaCl and incubated at 37 °C for 10 min. For each substrate concentration, a standard calibration was established for 2.5, 5, 7.5, and 10% cleavage by using the corresponding mixture of substrate and metabolite. The obtained fluorescence was then reported to each standard calibration to quantify the amount of metabolite formed. The K_m values (mean of at least three independent assays in duplicate) were calculated using GraphPad Prism 4 software, and the k_{cat} was determined using the equation $k_{\text{cat}} = V_{\text{max}}/[E]$. Fluorescence was measured on a Berthold Twinkle LB 970 (λ_{ex} = 340 nm, λ_{em} = 405 nm, lamp energy = 10,000).

Specificity of PL553 toward LTA4H

The ability of PL553 to discriminate LTA4H from APN enzymatic activity was tested using the (1)-Ala- β -naphthylamide and PL553 peptide substrates at 40 μM with either APN from porcine kidney (0.33 mU/ml) or recombinant human LTA4H (0.6 $\mu\text{g}/\text{ml}$). The enzymatic reactions proceeded for 1 h at 37 °C in a final volume of 100 μl of 50 mM Tris–HCl (pH 7.4) or 50 mM Tris–HCl (pH 7.4) and 100 mM NaCl for APN or LTA4H, respectively. Cleavage of PL553 (40 μM) by other aminopeptidases such as aminopeptidase A (APA), APB, leucine aminopeptidase (LAP), endoplasmic reticulum aminopeptidase-1 (ERAP-1), endoplasmic reticulum aminopeptidase-2 (ERAP-2), and aminopeptidase P (APP) was also investigated. The enzymes were tested in the following conditions: LAP (Sigma, 300 mU/ml) in 25 mM Tris–HCl buffer (pH 8.0); APA (R&D Systems, 0.1 $\mu\text{g}/\text{ml}$) in 25 mM Tris–HCl buffer (pH 8.0), 50 mM CaCl_2 , 200 mM NaCl, and 0.05% Triton X-100; APB (purified recombinant enzyme, a kind gift from T. Foulon, 450 $\mu\text{g}/\text{ml}$) in 50 mM Tris–HCl buffer (pH 7.4); ERAP-1 (R&D Systems, 0.5 mg/ml) in 25 mM Tris–HCl (pH 8.0); ERAP-2 (R&D Systems, 0.5 mg/ml) in 100 mM Mes (pH 6.5); and APP in 50 mM Tris (pH 7.0). The specificity of PL553 toward LTA4H was further tested against a series of other metalloproteases (ECE-1, neutral endopeptidase

[NEP], and angiotensin-converting enzyme [ACE]) in either 100 mM Hepes buffer (pH 6.8), 50 mM Tris (pH 7.4), or 50 mM Tris (pH 8.0) and NaCl (1%) for each enzyme, respectively, as well as against the serine fatty acid amide hydrolase (human recombinant FAAH, Cayman Chemical, 18.7 U/ml) in 50 mM Tris (pH 9.0) and 1 mM ethylenediaminetetraacetic acid (EDTA). The reactions were performed in a final volume of 100 μl and incubated for 1 h at 37 °C. At the end of the reaction, fluorescence was measured on a Berthold Twinkle LB 970 fluorimeter (λ_{ex} = 340 nm, λ_{em} = 405 nm, lamp energy = 10,000). For each enzyme tested, a positive control using a specific substrate was also performed in parallel (not shown).

Limits of detection and quantitation of LTA4H using PL553

Decreasing concentrations of recombinant LTA4H (1.0–0.05 $\mu\text{g}/\text{ml}$) were incubated with 40 μM PL553 for 5 h at 37 °C in 100 μl of 50 mM Tris–HCl (pH 7.4) and 100 mM NaCl buffer. The reaction was performed in black 96-well microplates and read on a Berthold Twinkle LB 970 (λ_{ex} = 340 nm, λ_{em} = 405 nm, lamp energy = 10,000). Repeated measures of PL553 substrate diluted to its final assay concentration in 50 mM HCl (pH 7.4) and 100 mM NaCl reaction buffer and incubated at 37 °C were performed. Fluorescence was read as described above. The PL553 limit of detection (LOD) value was calculated using $\text{LOD} = \text{mean} + 3\text{SD}$, where mean corresponds to the mean value of the signal produced by the substrate diluted in reaction buffer and SD corresponds to the standard deviation from that mean signal [25]. The obtained LOD value was then reported on the LTA4H standard curve to obtain the lowest detectable enzyme concentration. The limit of quantitation (LOQ) of the PL553 assay was obtained using the statistical method, in which its value is set at 10 standard deviations above the mean blank value. The obtained LOQ was reported on the LTA4H standard curve so as to obtain the lowest quantifiable LTA4H concentration.

Inhibitory potency measurements

To determine the in vitro inhibitory potencies or K_i values of different inhibitors toward LTA4H, 0.6 $\mu\text{g}/\text{ml}$ recombinant enzyme was preincubated for 30 min at 37 °C in 50 mM Tris–HCl (pH 7.4) and 100 mM NaCl, with increasing concentrations of inhibitor (from 10^{-10} to 10^{-4} M final concentration). The fluorescent substrates PL553 (40 μM) and (1)-Ala- β -naphthylamide (1 mM) were added in a final volume of 100 μl and incubated at 37 °C for 15 min. The fluorescent values were measured on a Berthold Twinkle LB 970 (λ_{ex} = 340 nm, λ_{em} = 405 nm, lamp energy = 10,000). Samples with 0% hydrolysis were obtained by adding the substrate to the buffer, and samples with 100% relative activity were prepared without the inhibitor. The percentage of cleavage was evaluated and compared with 100% relative activity, and the IC_{50} values were determined accordingly. The K_i values of the inhibitors (mean of at least three independent assays in duplicate) were calculated using the equation $K_i = \text{IC}_{50}/(1 + [S]/K_m)$, assuming their mode of binding to be competitive.

Inhibitory potencies toward APN were evaluated according to the same protocol but using (1)-Ala- β -NA (50 μM) in 50 mM Tris–HCl (pH 7.4). H-Glu-AMC (Bachem) was used at a concentration of 10 μM in 25 mM Tris–HCl (pH 8.0), 50 mM CaCl_2 , 200 mM NaCl, and Triton (0.05%) to measure APA inhibition (20 ng/ml, human recombinant, R&D Systems). APP inhibition (10 ng/ml, human recombinant, R&D Systems) was measured in 50 mM Tris (pH 7.4) and using the H-Lys-(Abz)-Pro-Pro-pNA substrate (20 μM , Bachem). LAP (200 $\mu\text{g}/\text{ml}$, ICN) was used in 25 mM Tris (pH 8.0) and with L-Leu- β -NA (100 μM , Sigma). NEP inhibition (100 ng/ml, rabbit kidney) was measured using Suc-Ala-Ala-Phe-AMC

substrate (20 μ M, Bachem) as described previously [26]. The fluorescent signal from 7-amido-4-methylcoumarin (AMC)-containing substrates was measured as described using the Berthold apparatus ($\lambda_{\text{ex}} = 340$ nm, $\lambda_{\text{em}} = 460$ nm, lamp energy = 10,000).

Ex vivo LTA4H activity

The ME pro-drugs of compounds **2** to **4** were each administered to three male OF1 mice (Charles River Laboratories, France) at 30 mg/kg intravenously in ethanol/Tween 80/water (1:1:8, v/v/v), whereas three other mice received vehicle alone (controls). Animals were euthanized 15 min postinjection, and their spleens and lungs were dissected out, rinsed with physiological serum, and stored at -80°C . On the day of the experiment, the organs were weighed and homogenized in 50 mM Tris-HCl (pH 6.8) with 100 μ M phenylmethanesulfonyl fluoride (PMSF) and 100 μ M leupeptin (50 mg tissue/ml) using a polytron. The tissue homogenates were then centrifuged for 30 min at 5000 rpm at 4°C , and the supernatants were collected. The total protein concentrations were quantified using the Bradford method. The tissue LTA4H enzymatic activity was assessed using either 1 mM L-Ala- β -NA or 40 μ M **PL553** using 400 μ g/ml protein. Assays were incubated for 5 h at 37°C , and fluorescence was measured on a Berthold Twinkle LB 970 fluorimeter ($\lambda_{\text{ex}} = 340$ nm, $\lambda_{\text{em}} = 405$ nm, lamp energy = 10,000). For each assay, total activity (TA) was measured in the presence of tissue and substrate alone, whereas nonspecific activity (NSA) was measured in the presence of an excess of LTA4H inhibitor (10 μ M kelatorphan) or with the same amount of a heat-denatured protein preparation (10 min at 70°C). The specific activity (SA) of LTA4H is obtained by subtracting the measured nonspecific activity (using inhibitor) from the total activity ($\text{SA} = \text{TA} - \text{NSA}$). These specific activities were then transformed in percentages taking the specific activity of the control animal as 100%. Statistical analyses (Student's *t* test) were performed using GraphPad Prism 4. The FAAH enzymatic activity contained in the same tissue preparations was assessed using 40 μ M AMC arachidonoyl amide (Cayman Chemical) in 50 mM Tris (pH 9.) and 1 mM EDTA. Fluorescence was read after 5 h at 37°C ($\lambda_{\text{ex}} = 340$ nm, $\lambda_{\text{em}} = 460$ nm, lamp energy = 10,000), and the FAAH specific activity was calculated by subtracting the base fluorescence from the substrate, measured in the same conditions, from the total activity.

Measurement of LTB4 levels by enzyme immunoassay

Endogenous LTB4 was extracted from the mice spleen cytosolic preparations using SEP-PAK C18 ICC cartridges (Waters), concentrated under reduced pressure and reconstituted in Tris buffer, supplied in the LTB4 immunoassay kit (Sapphire Bioscience). LTB4 concentrations were then measured following the recommendations of the manufacturer.

Results

Peptide substrate synthesis and screening

The synthetic pathway used for the preparation of the different α -aminoacyl- β -naphthylamides **1a** to **1f** is reported in [Supplemental Fig. 1 of the supplementary material](#). These compounds correspond to optically pure molecules of (S) configuration. They were initially screened using both LTA4H and APN in the same conditions and in comparison with the commonly used nonselective substrate Ala- β -NA. As shown in [Fig. 1](#), LTA4H preferentially cleaved compound **1d**, followed by **1a** and **1f**, whereas **1b** was barely cleaved and **1c** and **1e** were not substrates. For APN, only **1a** and **1b** appeared to be cleaved by APN, albeit much less

efficiently than Ala- β -NA. Thus, this rapid screening revealed that peptide **1d** [(L)-(4-benzoyl)Phe- β -NA] was efficiently and selectively cleaved by LTA4H as compared with APN. Therefore, this substrate designated as **PL553** was kept for further development.

Spectrofluorimetric characteristics of PL553

The UV spectrum of the **PL553** substrate revealed a maximum absorption (λ_{max}) of 210 nm ($\epsilon = 209,090 \text{ M}^{-1} \text{ cm}^{-1}$) (not shown). The fluorescent spectra obtained after excitation at 340 nm of the **PL553** substrate and of its fluorescent cleavage product, β -NA, show that they both display a single emission maximum ($\lambda_{\text{em}} = 410$ nm). At that maximum, β -NA emits a fluorescent signal of nearly 1000 relative fluorescent units (RFU), whereas the **PL553** substrate emits a signal of approximately 56 RFU for a signal-to-noise ratio of 16 ([Fig. 2](#)).

Enzymatic activities

Because of the discrepancy in the literature concerning the conditions in which the aminopeptidase activity of LTA4H should be tested, particularly concerning the pH, experiments were performed to determine the optimal pH of LTA4H aminopeptidase activity using **PL553**. Moreover, to confirm the activation of LTA4H by chloride ions [27], this study was performed in the presence or absence of 100 mM NaCl. Results of these experiments reveal that, in Tris-HCl buffer, human recombinant LTA4H cleaved **PL553** at an optimal pH of 6.8, whereas the addition of NaCl induced a slight shift in that optimum, which displayed a plateau between 7.0 and 7.5 ([Fig. 3A](#)). The enzymatic activity was increased in the presence of 100 mM NaCl, particularly between pHs 7.0 and 8.0 ([Fig. 3A](#)). Overall, these results prompted the use of 50 mM Tris-HCl (pH 7.4) and 100 mM NaCl as LTA4H reaction buffer for the **PL553** assay.

Kinetic parameters and selectivity of PL553

Using the conditions set forth, the kinetic parameters of **PL553** toward LTA4H were determined. Results reveal that the substrate binds to the active site of the enzyme with a good apparent affinity, expressed by its K_m of $16 \pm 3 \mu\text{M}$, but that it is slowly cleaved with a k_{cat} of $0.62 \pm 0.08 \text{ s}^{-1}$ ([Fig. 3B](#)). Nevertheless, with a catalytic efficiency (k_{cat}/K_m) of $3.8 \pm 0.5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, **PL553** appears to be a much better LTA4H substrate than (L)-Ala- β -naphthylamide ($4.09 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$). In addition to APN, **PL553** was further shown to be resistant to cleavage by other aminopeptidases (APA, APB, ERAP-1, and ERAP-2 of the M1 family as well as LAP of the M17 family and APP of the M24 family of metalloproteases) ([Fig. 3C](#)). Interestingly, **PL553** was cleaved by FAAH (not shown), a serine hydrolase involved in the degradation of numerous bioactive fatty acid amides [28].

In vitro characteristics of PL553 assay

Using a concentration of 40 μM **PL553** and decreasing concentrations of LTA4H, the fluorescent signal was found to be linear over a 2-h incubation for low concentrations of enzyme (i.e., up to 0.4 $\mu\text{g/ml}$), with the reaction being complete after 5 h ([Fig. 4A](#)). Linearization of the fluorescence values obtained after an incubation of 2 h (not shown) or 5 h yielded linear calibration curves ($R^2 = 0.97$). After 5 h, linearization of the lowest enzyme concentrations shows an LOD of the **PL553** assay of 0.008 $\mu\text{g/ml}$ enzyme and an LOQ of 0.02 $\mu\text{g/ml}$ LTA4H, corresponding to 2 ng of enzyme in the assay ([Fig. 4B](#)). The LOD and LOQ of the assay after only 2 h of incubation were 0.02 and 0.05 $\mu\text{g/ml}$, respectively (not shown).

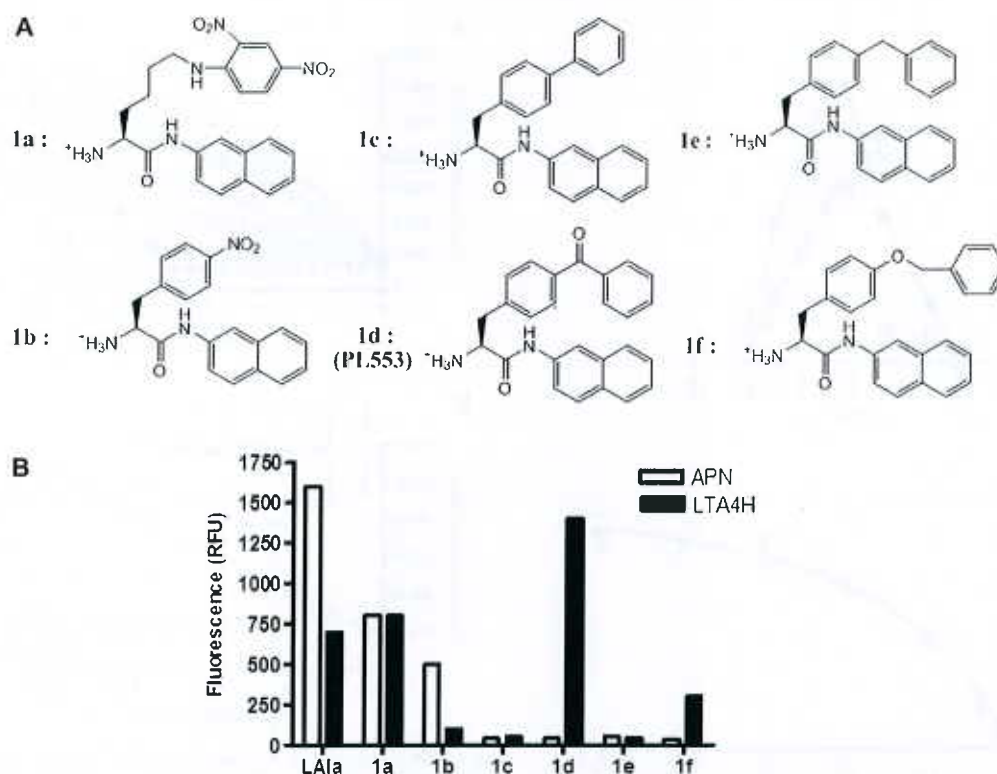


Fig. 1. (A) Structures of the different α -aminoacyl- β -naphthylamide compounds tested as potential LTAH substrates. (B) Fluorescent signal produced in the presence of APN or LTA4H was measured in parallel as described using a 20- μ M concentration of either (L)-Ala- β -NA (LAla) or compounds 1a to 1f. Reactions were left to proceed for 1 h at 37 °C, and the signal was measured on a Berthold Twinkle fluorimeter (λ_{ex} = 340 nm, λ_{em} = 405 nm, lamp energy = 10,000).

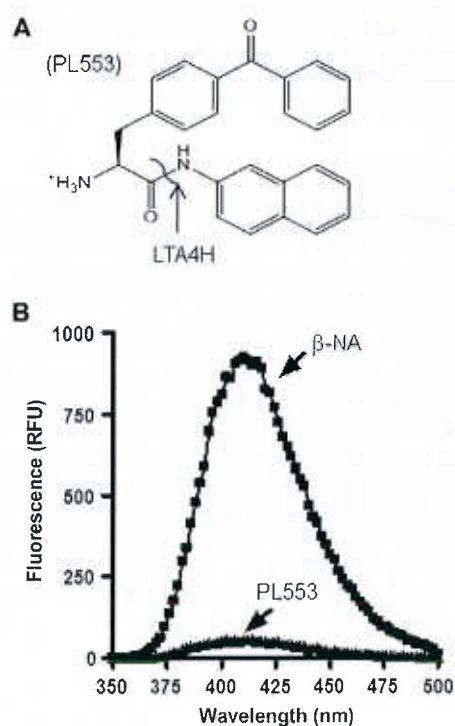


Fig. 2. (A) LTA4H (human recombinant) cleaves PL553 at its amide bond, releasing the nonfluorescent moiety L-(4-benzoyl)phenylalanine from the fluorescent β -NA. (B) Emission spectra of the PL553 substrate (■) and of the β -NA metabolite (▲), each diluted to 10 μ M in 50 mM Tris-HCl (pH 7.4) and 100 mM NaCl and measured on a PerkinElmer fluorimeter at 340 nm.

Inhibitory potencies

To further validate the use of the novel PL553 assay in vitro, the inhibitory potencies of known competitive inhibitors were determined in parallel using PL553 and the reference (L)-Ala- β -NA substrate (Table 1). Results show that the same potencies are obtained using either substrate, validating the use of the currently designed specific substrate for LTA4H enzymatic activity. Thus, whereas PC18 (methionine thiol) [29], a specific APN inhibitor, bound LTA4H with only moderate micromolar apparent affinity (K_i = 577 ± 33 μ M), kelatorphan [30] and the recently published glutamic acid analog, compound 2 [2-amino-5-(4-(benzyloxy)phenylamino)-5-oxopentanoic acid], inhibited LTA4H with K_i values in the nanomolar range, as described previously [24] (Fig. 5 and Table 1).

Thereafter, new amino-phosphinic compounds 3 to 5, designed as potential inhibitors of LTA4H, were also tested and shown to inhibit the enzyme with K_i values in the nanomolar range, with the two best compounds being 3 and 4, displaying K_i values of approximately 5 nM (Table 2). Using PL553, the ME forms of these compounds were found to possess K_i values of 78.7 ± 0.3 and 15.6 ± 0.2 , respectively (not shown). Selectivity of these novel compounds toward other metalloproteases was also investigated. Interestingly, all three inhibitors appeared to be specific toward LTA4H in that they did not bind other aminopeptidases such as APN, APA, APP, and LAP or NEP, which, for instance, binds kelatorphan with high affinity (Table 2).

Ex vivo LTA4H activity

The (L)-Ala- β -NA and PL553 assays were compared for the evaluation of LTA4H activity using ex vivo tissue preparations. For this

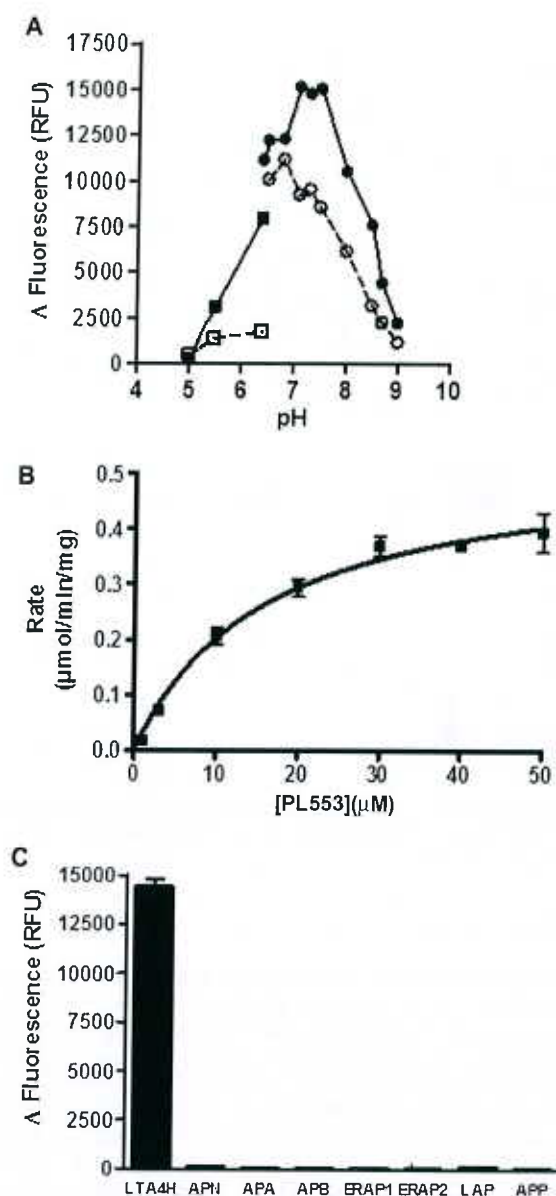


Fig. 3. (A) Determination of the optimal pH of LTA4H using **PL553** (40 μ M) in 50 mM Mes (pHs 5.0 and 6.0, \square and \blacksquare) or 50 mM Tris-HCl (\bullet and \circ), supplemented (full line and symbols) or not (dotted line and open symbols) with 100 mM NaCl. (B) Kinetic parameters of the **PL553** substrate. Increasing concentrations of **PL553** were incubated for 10 min at 37 $^{\circ}$ C in 50 mM Tris-HCl (pH 7.4) and 100 mM NaCl containing 0.6 μ g/ml LTA4H. The K_m of **PL553** was 16 ± 3 μ M, and the catalytic constant k_{cat} was 0.62 ± 0.09 s $^{-1}$, leading to a specificity constant (k_{cat}/K_m) of $3.8 \pm 0.5 \times 10^4$ M $^{-1}$ s $^{-1}$. (C) Specificity study of **PL553** versus various aminopeptidases. **PL553** (40 μ M) was incubated with the different aminopeptidases at 37 $^{\circ}$ C for 1 h as described, and the fluorescent signal was read on a Berthold Twinkle (λ_{ex} = 340 nm, λ_{em} = 405 nm, lamp energy = 10,000). Deltas (Δ) of fluorescence represent the signal measured in the presence of a given enzyme minus the signal of the substrate diluted in buffer alone.

purpose, male mice were injected intravenously with either vehicle or the ME form of compound **2** [24] as well as the ME forms of the two most potent inhibitors synthesized in this study, that is, compounds **3** and **4**. To best maintain protein integrity, as well as to limit the expression of contaminating activities, the cytosolic fractions were prepared in the presence of leupeptin as well as the irreversible serine protease inhibitor PMSF. This, combined with the addition of an LTA4H inhibitor (here kelatorphan or EDTA) to measure nonspecific activity, allowed for the nearly complete lack

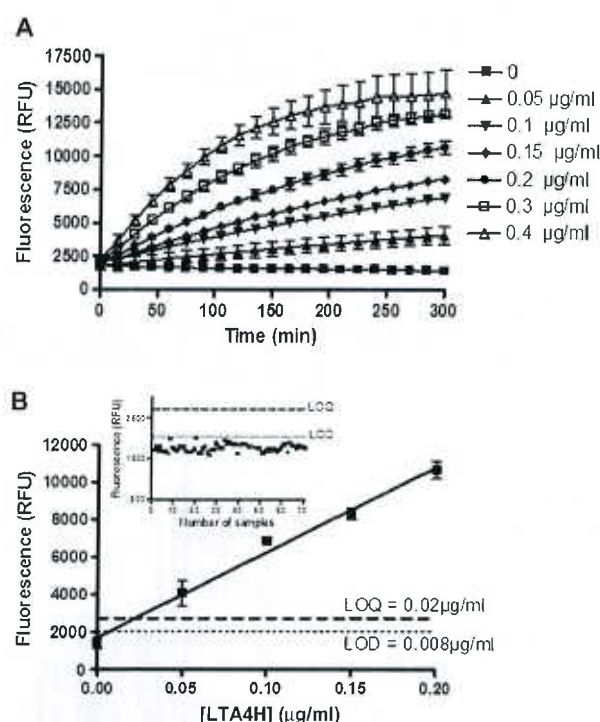


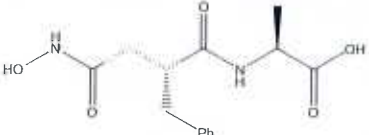
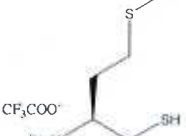
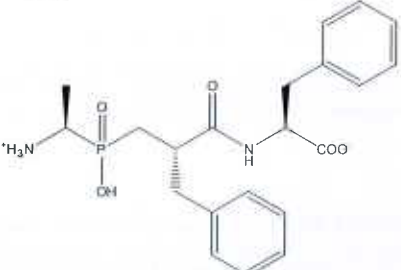
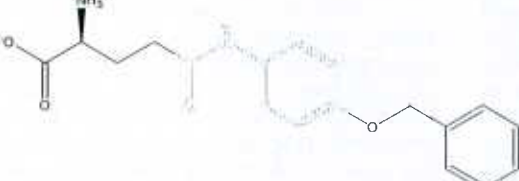
Fig. 4. LOD and LOQ values of LTA4H using **PL553**. (A) Decreasing concentrations of LTA4H were incubated with 40 μ M **PL553** for 5 h at 37 $^{\circ}$ C in a Berthold Twinkle fluorimeter (λ_{ex} = 340 nm, λ_{em} = 405 nm, lamp energy = 10,000), and the fluorescent signal was measured every 15 min. The LTA4H concentrations used were 0.4 (Δ), 0.3 (\square), 0.2 (\bullet), 0.15 (\blacklozenge), 0.1 (\blacktriangledown), and 0.05 (\blacktriangle) μ g/ml. \blacksquare , Fluorescence of the peptide substrate alone. (B) **PL553** was diluted at a final concentration of 40 μ M in a final volume of 100 μ l of 50 mM Tris-HCl (pH 7.4) and 100 mM NaCl reaction buffer. Repeated measures of the signal emitted by these diluted solutions were recorded and used to determine the LOD and LOQ values of this substrate. An LTA4H standard curve was obtained by plotting the results obtained after 5 h of incubation at 37 $^{\circ}$ C with **PL553** (40 μ M). The LOD and LOQ values of the substrate were reported on the standard curve to obtain the lowest detectable and quantifiable LTA4H concentrations.

of contaminating activity, including the serine protease FAAH activity (Supplemental Fig. 3). The ex vivo inhibitory potency of the ME form of compound **2**, previously shown to cross the plasma membrane [25], was measured in the spleen cytosolic fraction from control mice and found to display a K_i of 17.8 ± 0.04 nM using the **PL553** assay (not shown). Moreover, using the same cytosolic fractions from two tissues known to express LTA4H, lung and spleen, its activity measured with (L)-Ala- β -NA did not appear to be significantly inhibited by compound **2** to **4** ME administration (Fig. 6A). However, when the same ex vivo experiment was performed using the **PL553** assay, a significant inhibition of the enzymatic activity measured in the tissues, ranging from 60 to 80%, was observed (Fig. 6B and Supplemental Fig. 4). Interestingly, quantification of endogenous LTB₄, the metabolite resulting from LTA4H activity by enzyme immunoassay, revealed parallel significant decreased levels of the leukotriene as compared with controls (Fig. 6C).

Discussion

LTA4H belongs to the M1 family of zinc-dependent aminopeptidases and possesses structural and functional similarities with APN. More detailed analysis of their 3D structures, however, provided evidence of a main difference that could be advantageously used to design selective substrates for each peptidase. In LTA4H,

Table 1
Potency of LTA4H inhibitors measured with PL553 or (L)-Ala-β-Naphthylamide.

Compound	Structure	K_i (nM)		
		PL553	L-Ala-β-NA	Literature
Kelatorphan		5.48 ± 0.33	7.36 ± 1.33	$K_i = 10$ [37]; $K_i = 9 ± 2.5$ [39]
PC18		577 ± 33	1382 ± 380	
PL250		1.40 ± 0.34	1.07 ± 0.37	
Compound 2		16.20 ± 0.33	21 ± 4	$IC_{50} = 20$ [24]

Note: The fluorogenic substrate PL553 (40 μM) was used to evaluate the potencies of known inhibitors toward LTA4H and compared with the results obtained with (L)-Ala-β-NA (1 mM). K_i values were obtained from data shown in Fig. 5 using the Cheng and Prusoff equation [$K_i = IC_{50}/(1 + [S]/K_m)$].

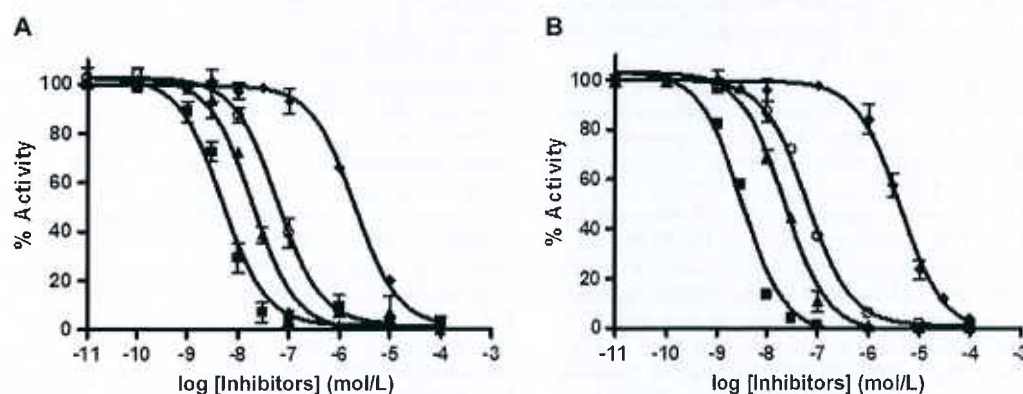
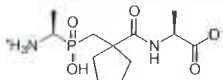
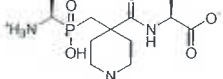
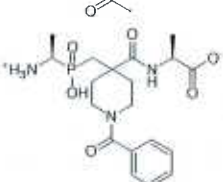


Fig. 5. Inhibitory potencies of known inhibitors of LTA4H using PL553 and (L)-Ala-β-NA. (A and B) Use of PL553 (40 μM) in panel A was compared with that of (L)-Ala-β-NA (1 mM) in panel B to evaluate the inhibitory potencies of PL250 (■), kelatorphan (▲), compound 2 (○), and PC18 (◆). Decreasing concentrations of inhibitors were diluted in 50 mM Tris-HCl (pH 7.4) and 100 mM NaCl with 0.6 μg/ml LTA4H and preincubated at 37 °C for 30 min. Reactions were then started by the addition of either substrate and incubated for 10 min at 37 °C, and the fluorescent signal was read as described. The obtained K_i values from the conversion of the obtained IC_{50} values assuming competitive inhibition of studied inhibitors are reported in Table 1.

the S_1 subsite is very deep, binding the long lipid chain of LTA4 or the 4-benzyloxyphenyl side chain of inhibitors such as compound 2 [22,24]. In *Escherichia coli* APN, binding to the S_1 subsite is hindered by the presence of a methionine residue in position 260 [31,32], which in human APN is replaced by an alanyl residue (Ala351), leading to a larger subsite [33] optimally occupied by a phenethyl group [34,35].

Using these data, we designed new fluorescent substrates containing different aromatic and polyaromatic P_1 side chains and compared their cleavage by either APN or LTA4H (Fig. 1). Of these, only two compounds appeared to be specifically cleaved by LTA4H, compounds 1f and 1d (PL553), with the latter producing by far the most intense fluorescent signal (Fig. 1). The low signal produced by 1f can probably be attributed to its P_1 side chain, which is also

Table 2
Inhibitory potencies of three novel compounds toward LTA4H and related metalloproteases.

Compound	Structure	K_i (nM)					
		LTA4H	APN	APA	APP	LAP	NEP
Compound 3		4.6 ± 0.03	223 ± 2	>10,000	>10,000	>10,000	>10,000
Compound 4		5.3 ± 0.01	2360 ± 60	>10,000	>10,000	>10,000	>10,000
Compound 5		31.2 ± 0.9	>10,000	>10,000	>10,000	>10,000	>10,000

Note: The inhibitory potency of each compound was evaluated using 40 μ M **PL553** for LTA4H, 50 μ M L-Ala- β -NA for APN, 10 μ M H-Glu-AMC for APA, 20 μ M H-Lys-(Abz)-Pro-Pro-pNA for APP, 100 μ M L-Leu- β -NA for LAP, and 20 μ M Suc-Ala-Ala-Phe-AMC for NEP. The inhibitors did not bind FAAH (not shown). K_i values were calculated from IC_{50} values using the Cheng and Prusoff equation [$K_i = IC_{50}/(1 + [S]/K_m)$].

found in very potent inhibitors of LTA4H [24]. Thus, it may well be that cleavage of this substrate leads to a nonfluorescent metabolite with high affinity for the enzyme, inhibiting the enzymatic reaction. Moreover, it is interesting to note that compounds **1d** and **1e**, differing only at the junction between the two aromatic cycles of their P_1 side chains, show such great differences in their catalytic properties. To understand the high impact of this junction on LTA4H recognition, these compounds were docked in the 3D structure of LTA4H (PDB ID: 1HS6) using GOLD software [36]. This docking revealed that the backbones of the two molecules are perfectly superimposed in the active site, although compound **1d** had the capacity to form a stabilizing hydrogen bond between the carbonyl of its P_1 side chain and the NH of Ala137 of the S_1 subsite of LTA4H. This observation, combined with the lower flexibility of compound **1d** as compared with compound **1e**, likely provides compound **1d** with the better catalytic properties (Fig. 7).

Study of the kinetic parameters of **1d**, **PL553**, toward LTA4H revealed a Michaelis–Menten constant (K_m) of approximately 16 μ M, a constant bettered 30-fold compared with the commonly used Ala- β -NA ($K_m = 499 \pm 72 \mu$ M) fluorescent substrate. Moreover, the catalytic efficiency of **PL553** (k_{cat}/K_m) was found to be $3.8 \pm 0.5 \times 10^4 s^{-1} M^{-1}$, that is, superior to that of Ala- β -NA by approximately 10-fold (Fig. 3). More interesting, the **PL553** substrate, when tested against a panel of aminopeptidases, was found to be highly selective for LTA4H (Fig. 3C). Even though the substrate was also cleaved by the FAAH, revealing the ability of **PL553** to mimic the lipidic-type substrates derived from arachidonic acid, its activity was completely inhibited by the addition of a nonspecific serine protease inhibitor in the assay. **PL553** was also resistant to cleavage by other related metallo-endoproteases from the MA clan, such as neprilysin and ACE (not shown), further underlying its selectivity.

Kinetic analysis of **PL553** cleavage over 5 h shows the enzyme concentration and time dependency of the fluorescent signal and reveals that dose–response curves for LTA4H can be obtained rapidly, within 1 h, or in up to 5 h, depending on the enzyme concentration sought. After 5 h, the **PL553** assay reached the lowest LOD (0.008 μ g/ml) and LOQ (0.02 μ g/ml), corresponding to 0.8 and 2 ng per assay, respectively, reflecting its high sensitivity conferred by the good specificity constant of the substrate. Use of the **PL553** assay for in vitro inhibitory potency determination was validated by

comparing the results obtained using the assay in parallel with the L-Ala- β -NA assay. Results of these experiments reveal that the K_i values obtained using **PL553** are comparable to those obtained using L-Ala- β -NA (Fig. 5 and Table 1) as well as those reported previously using the epoxide assay. Indeed, for example, kelatorphan ($K_i = 6$ nM in this study) was initially reported to inhibit the epoxide activity with an IC_{50} of 5 nM and of 7 nM using the aminopeptidase assay [37] and more recently with K_i values of 11 and 9 nM, respectively [38]. Moreover, compound **2**, reported to display an IC_{50} value of 20 nM [25] using the aminopeptidase assay, was found here, using **PL553**, to possess a K_i of approximately 16 nM (Fig. 5 and Table 1). Although compound **2** was shown to be inactive in the whole blood LTA4 hydrolase assay, its ME form displayed an IC_{50} of 48 nM, revealing its epoxide cleavage inhibiting potency related to its capacity to cross the cell membrane. Interestingly, the **PL553** assay performed in the presence of a tissue preparation expressing LTA4H allowed the determination of an ex vivo K_i for the ME form of compound **2** (17.8 nM) (not shown).

Taking into account the ability of some amino-phosphinic compounds designed for the inhibition of APN [34] to bind the LTA4H active site at the level of its S'_1 and S'_2 subsites [38], chemical modifications of these molecules that could induce a selective LTA4H recognition were explored. The most interesting results were obtained with compounds containing saturated cyclic structures such as cyclopentyl or piperidinyl moieties at the P'_1 level (compounds **3–5**). Indeed, these molecules were found to inhibit LTA4H with low nanomolar potencies evaluated with the **PL553** assay at approximately 5 nM for compounds **3** and **4**, whereas compound **5** appeared to be slightly less potent, with a K_i of approximately 30 nM (Table 2). Moreover, the three compounds were selective for LTA4H in that they did not inhibit APA, APP, or LAP aminopeptidases ($IC_{50} > 10^{-5}$ M) (Table 2). Although compound **3** could bind APN with an affinity of 223 ± 2 nM, it remained nearly 50 times more potent against LTA4H. Changing the cyclopentyl moiety of compound **3** to a more cumbersome piperidinyl moiety (compound **4**) did not alter the potency toward LTA4H but had a great impact on binding to APN, reducing the inhibitory potency of the resulting molecule to approximately 2.4 μ M and suggesting that LTA4H inhibitor specificity lies within its S'_1 subsite.

To demonstrate the usefulness of the **PL553** assay in the investigation of LTA4H physiological functions and in the development

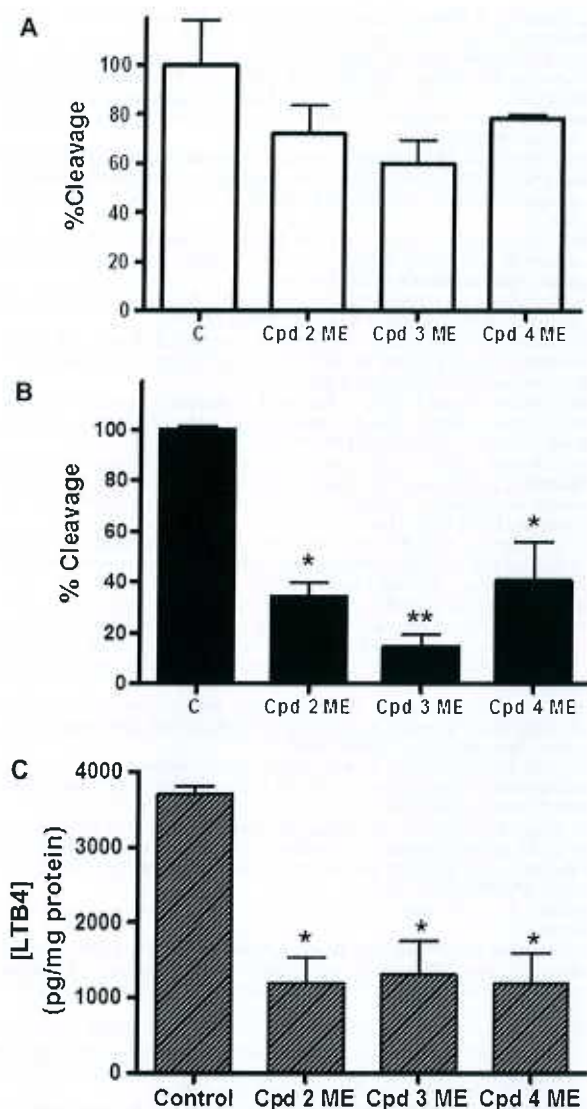


Fig. 6. In vivo inhibition of LTA4H-related LTB4 formation. ME derivatives of compounds (Cpd) 2 to 4 were administered to male mice at 30 mg/kg intravenously, and the spleens were dissected 15 min after injection and stored at -80°C . Controls (C) correspond to intravenous injection of the same volume of vehicle without inhibitors. The tissues were homogenized and the LTA4H activity was quantified in 400 $\mu\text{g}/\text{ml}$ of a cytosolic fraction using either (L)-Ala- β -NA (1 mM) (A) or **PL553** (40 μM) (B) in 100 μl of 50 mM Tris (pH 7.4) and 100 mM NaCl. (C) Tissue LTB4 concentrations were assayed in parallel in the cytosolic fraction by enzyme immunoassay. * $P < 0.05$ and ** $P < 0.01$ versus control; Student's t test.

of drugs targeting its activity, we performed in vivo experiments using the previously published compound **2** as a positive control as well as two novel, specific phosphinic compounds, **3** and **4**, described here. For these experiments, for the drugs to cross the plasma membranes of the LTA4H-expressing cells [25], the ME pro-drug forms of the three molecules were used. Results of these experiments displayed in Fig. 6 (combined with those of Supplemental Figs. 3 and 4) show that the **PL553** assay is the first and only assay able to selectively detect and quantify endogenous activity of LTA4H as well as to assess the impact of the administration of targeted drugs in animal tissues. This is demonstrated by the observed parallel inhibitor-induced decrease of the aminopeptidase activity as well as of the endogenous pro-inflammatory LTB4 leukotriene levels produced by the epoxide hydrolase activity of LTA4H. Taken together, these results demonstrate the link between

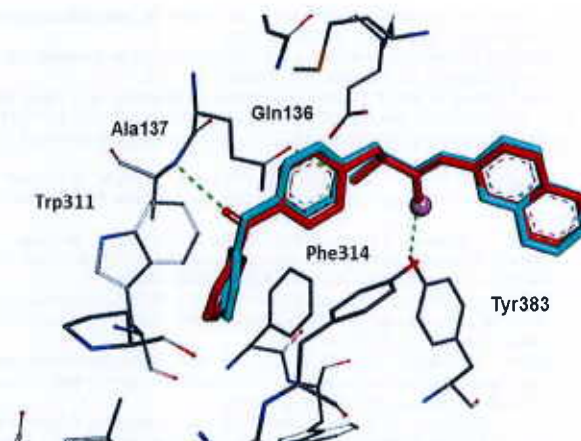


Fig. 7. Superimposition of compounds **1d** (red) and **1e** (turquoise) inside the S_1 subsite of LTA4H (PDB ID: 1HS6) using GOLD software [6]. The catalytic Zn^{2+} is in purple. The carbonyl of the benzophenone side chain of **1d** is hydrogen bonded of the NH of Ala137. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the aminopeptidase activity of the enzyme and its hydrolase activity, and they underlie the critical role of the S_1 side chain occupation of the enzyme by **PL553**.

In conclusion, the design of **PL553**, the first selective LTA4H substrate, leads to a novel LTA4H enzymatic assay with unique high specificity and sensitivity, providing a useful tool for the assessment of LTA4H activity in vivo. This novel assay can also be used on different cell fractions or extracts to help identify the subcellular expression pattern of this enzyme. Indeed, LTA4H is thought to produce LTB4 at the intracellular level, more precisely in the cytosol of LTA4H-expressing cells. However, although the recent identification of an endogenous tripeptide substrate of LTA4H also unravels a physiological basis for the observed aminopeptidase activity of the enzyme [15], questions still remain as to the exact subcellular location of this activity because the identified Pro-Gly-Pro substrate is located extracellularly [15]. Although this could be in agreement with the reported expression of a soluble LTA4H in human plasma or bronchoalveolar lavage, its activity there still remains to be clearly demonstrated [15]. Finally, as shown in this study, the **PL553** assay provides the only specific tool for the simple and rapid screening of inhibitors of LTA4H-induced leukotriene formation as well as for the study of their impact in inflammatory diseases, for example, providing a link between an observed pharmacological effect and an in vivo inhibition of enzymatic activity.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ab.2013.06.016>.

References

- [1] J.Z. Haeggström, Structure and regulation of leukotriene A4 hydrolase, *Am. J. Respir. Crit. Care Med.* 161 (2000) S25–S32.
- [2] J.Z. Haeggström, A. Wetterholm, Enzymes and receptors in the leukotriene cascade, *Cell. Mol. Life Sci.* 59 (2002) 742–753.

- [3] M.Q. Zhang, Leukotriene A4 hydrolase: a new target for anti-inflammatory intervention, *Curr. Med. Chem.* 4 (1997) 67–78.
- [4] T.D. Penning, Inhibitors of leukotriene A4 (LTA4) hydrolase as potential anti-inflammatory agents, *Curr. Pharm. Des.* 7 (2001) 163–179.
- [5] X. Chen, S. Wang, N. Wu, C.S. Yang, Leukotriene A4 hydrolase as a target for cancer prevention and therapy, *Curr. Cancer Drug Targets* 4 (2004) 267–283.
- [6] A.W. Ford-Hutchinson, Leukotriene B4 in inflammation, *Crit. Rev. Immunol.* 10 (1990) 1–12.
- [7] J.Z. Haeggström, F. Kull, P.C. Rudberg, F. Tholander, M.M.G.M. Thunnissen, Leukotriene A4 hydrolase, *Prostaglandins Other Lipid Mediat.* 68 (69) (2002) 496–510.
- [8] C.D. Funk, O. Rådmark, J.Y. Fu, T. Matsumoto, H. Jörnvall, T. Shimizu, B. Samuelsson, Molecular cloning and amino acid sequence of leukotriene A4 hydrolase, *Proc. Natl. Acad. Sci. U. S. A.* 84 (1987) 6677–6681.
- [9] J.F. Medina, O. Rådmark, C.D. Funk, J.Z. Haeggström, Molecular cloning and expression of mouse leukotriene A4 hydrolase cDNA, *Biochem. Biophys. Res. Commun.* 176 (1991) 1516–1524.
- [10] N. Makita, C.D. Funk, E. Imai, R.L. Hoover, K.F. Badr, Molecular cloning and functional expression of rat leukotriene A4 hydrolase using the polymerase chain reaction, *FEBS Lett.* 299 (1992) 273–279.
- [11] M. Minami, H. Mutoh, N. Ohishi, Z. Honda, H. Bito, T. Shimizu, Amino-acid sequence and tissue distribution of guinea-pig leukotriene A4 hydrolase, *Gene* 161 (1995) 249–251.
- [12] M. Minami, N. Ohishi, H. Mutoh, T. Izumi, H. Bito, H. Wada, Y. Seyama, H. Tob, T. Shimizu, Leukotriene A4 hydrolase is a zinc-containing aminopeptidase, *Biochem. Biophys. Res. Commun.* 173 (1990) 620–626.
- [13] J.Z. Haeggström, A. Wetterholm, B.L. Vallee, B. Samuelsson, Leukotriene A4 hydrolase: an epoxide hydrolase with peptidase activity, *Biochem. Biophys. Res. Commun.* 173 (1990) 431–437.
- [14] L. Orning, J.K. Gierse, F.A. Fitzpatrick, The bifunctional enzyme leukotriene-A4 hydrolase is an arginine aminopeptidase of high efficiency and specificity, *J. Biol. Chem.* 269 (1994) 11269–11273.
- [15] R.J. Snelgrove, P.L. Jackson, M.T. Hardison, B.D. Noerager, A. Kinloch, A. Gaggar, S. Shastry, S.M. Rowe, Y.M. Shim, T. Hussel, J.E. Blalock, A critical role for LTA4H in limiting pulmonary neutrophilic inflammation, *Science* 330 (2010) 90–94.
- [16] M.M.G.M. Thunnissen, P. Nordlund, J.Z. Haeggström, Crystal structure of human leukotriene A4 hydrolase, a bifunctional enzyme in inflammation, *Nat. Struct. Biol.* 8 (2001) 131–135.
- [17] J.Z. Haeggström, F. Tholander, A. Wetterholm, Structure and catalytic mechanisms of leukotriene A4 hydrolase, *Prostaglandins Other Lipid Mediat.* 83 (2007) 198–202.
- [18] J.Z. Haeggström, Cytosolic liver enzymes catalysing the hydrolysis of leukotriene A4 into leukotriene B4 and 5,6-dihydroxy-eicosatetraenoic acid, *Methods Enzymol.* 187 (1990) 324–334.
- [19] A.M. Liang, E. Claret, J. Ouled-Diaf, A. Jean, D. Vogel, D.R. Light, S.W. Jones, W.J. Guilford, J.F. Parkinson, R.M. Snider, Development of a homogeneous time-resolved fluorescence leukotriene B4 assay for determining the activity of leukotriene A4 hydrolase, *J. Biochem. Screen.* 12 (2007) 536–546.
- [20] F. Tholander, J. Haeggström, Assay for rapid analysis of the tri-peptidase activity of LTA4 hydrolase, *Proteins* 67 (2007) 1113–1118.
- [21] M.M.G.M. Thunnissen, B. Anderson, B. Samuelsson, C.-H. Wong, J.Z. Haeggström, Crystal structures of leukotriene A4 hydrolase in complex with captopril and two competitive tight-binding inhibitors, *FASEB J.* 16 (2002) 1648–1650.
- [22] J. Xiaolu, L. Zhou, D. Wei, H. Meng, Y. Liu, L. Lai, Activation and inhibition of leukotriene A4 hydrolase aminopeptidase activity by diphenyl ether and derivatives, *Bioorg. Med. Chem. Lett.* 18 (2008) 6549–6552.
- [23] I. Schechter, A. Berger, On the size of the active site in proteases: I. Papain, *Biochem. Biophys. Res. Commun.* 27 (1967) 157–162.
- [24] T.A. Kirkland, M. Adler, J.G. Bauman, M. Chen, J.Z. Haeggström, B. King, M.J. Kochanny, A.M. Liang, L. Mendoza, G.B. Phillips, M. Thunnissen, L. Trinh, M. Whitlow, B. Ye, H. Ye, J. Parkinson, W.J. Guilford, Synthesis of glutamic acid analogs as potent inhibitors of leukotriene A4 hydrolase, *Bioorg. Med. Chem.* 16 (2008) 4963–4983.
- [25] D.A. Armbruster, M.D. Tillman, L.M. Hubbs, Limit of detection (LOD)/limit of quantitation (LOQ): comparison of the empirical and the statistical methods exemplified with GC–MS assays of abused drugs, *Clin. Chem.* 40 (1994) 1233–1238.
- [26] M.G. Spillantini, A. Panconesi, P.L. Del Bianco, F. Scuteri, Enkephalinase and angiotensin converting enzyme activities in human venous and arterial plasma, *Neuropeptides* 8 (1986) 111–117.
- [27] A. Wetterholm, J.Z. Haeggström, Leukotriene A4 hydrolase: an anion activated peptidase, *Biochim. Biophys. Acta* 1123 (1992) 275–281.
- [28] B.F. Cravatt, D.K. Glang, S.P. Mayfield, D.L. Boger, R.A. Lerner, N.B. Gilula, Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides, *Nature* 384 (1996) 83–87.
- [29] M.C. Fournié-Zaluski, P. Coric, S. Turcaud, L. Bruetsch, E. Lucas, F. Noble, B.P. Roques, Potent and systemically active aminopeptidase N inhibitors designed from active-site investigation, *J. Med. Chem.* 35 (1992) 1259–1266.
- [30] M.C. Fournié-Zaluski, A. Coulaud, R. Bouboutou, P. Chaillet, J. Devin, G. Waksman, J. Costentin, B.P. Roques, New bidendates as full inhibitors of enkephalin-degrading enzymes: synthesis and analgesic properties, *J. Med. Chem.* 28 (1985) 1158–1169.
- [31] A. Addlagatta, L. Gay, B.W. Matthews, Structure of aminopeptidase N from *Escherichia coli* suggests a compartmentalized, gated active site, *Proc. Natl. Acad. Sci. U. S. A.* 103 (2006) 13339–13344.
- [32] K. Ito, Y. Nakajima, Y. Onohara, M. Takeo, K. Nakashima, F. Matsubara, T. Ito, T. Yoshimoto, Crystal structure of aminopeptidase N (proteobacteria alanyl aminopeptidase) from *Escherichia coli* and conformational change in methionine 260 involved in substrate recognition, *J. Biol. Chem.* 281 (2006) 33664–33676.
- [33] A.H. Wong, D. Zhou, J.M. Rini, The X-ray crystal structure of human aminopeptidase N reveals novel dimer and the basis for peptide processing, *J. Biol. Chem.* 287 (2012) 36804–36813.
- [34] H. Chen, B.P. Roques, M.C. Fournié-Zaluski, Design of the first highly potent and selective aminopeptidase N (EC 3.4.11.2) inhibitor, *Bioorg. Med. Chem. Lett.* 9 (1999) 1511–1516.
- [35] M. Drag, J. Grembecka, M. Pawelczak, P. Kafarski, α -Aminoalkylphosphonates as a tool in experimental optimisation of P1 side chain shape of potential inhibitors in S1 pocket of leucine and neutral aminopeptidases, *Eur. J. Med. Chem.* 40 (2005) 754–771.
- [36] GOLD 5.1, GOLD Suite, CCDC (Cambridge Crystallographic Data Centre) Software.
- [37] T.D. Penning, L.J. Askonas, S.W. Djuric, R.A. Haack, S.S. Yu, M.L. Michener, G.G. Krivi, E.Y. Pyla, Kelatorphan and related analogs: potent and selective inhibitors of leukotriene A4 hydrolase, *Bioorg. Med. Chem. Lett.* 5 (1995) 2517–2522.
- [38] F. Tholander, A. Muroya, B.P. Roques, M.C. Fournié-Zaluski, M.M.G.M. Thunnissen, J.Z. Haeggström, Structure-based dissection of the active site chemistry of leukotriene A4 hydrolase: implications for M1 aminopeptidases and inhibitor design, *Chem. Biol.* 15 (2008) 920–929.
- [39] F. Tholander, B.P. Roques, M.C. Fournié-Zaluski, M.M.G.M. Thunnissen, J.Z. Haeggström, Crystal structure of leukotriene A4 hydrolase in complex with kelatorphan: implications for design of zinc metallopeptidase inhibitors, *FEBS Lett.* 584 (2010) 3446–3451.