

Discovery of the Oral Leukotriene-C4 Synthetase Inhibitor (1S,2S)-2-({5-[(5-Chloro-2,4-difluorophenyl)(2-fluoro-2-methylpropyl)amino]-3-methoxypyrazin-2-yl}carbonyl)cyclopropanecarboxylic acid (AZD9898) as a New Treatment for Asthma

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Discovery of the Oral Leukotriene-C4 Synthetase Inhibitor (1*S*,2*S*)-2-({5-[(5-Chloro-2,4-difluorophenyl)(2-fluoro-2-methylpropyl)amino]-3-methoxypyrazin-2-yl}carbonyl)cyclopropanecarboxylic acid (AZD9898) as a New Treatment for Asthma.

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Abstract

While bronchodilators and inhaled corticosteroids are the mainstay of asthma treatment, up to 50% of asthmatics remain uncontrolled. Many studies show the cysteine leukotriene cascade remains highly activated in some asthmatics, even those on high dose inhaled or oral corticosteroids. Hence inhibition of LTC₄S enzyme could provide a new and differentiated core treatment for patients with a highly activated cysteine leukotriene cascade. Starting from a screening hit **3**, a program to discover oral inhibitors of LTC₄S led to AZD9898 (**36**), a picomolar LTC₄S inhibitor (IC₅₀ = 0.28 nM) with high lipophilic ligand efficiency (LLE = 8.5), which displays nanomolar potency in cells (PBMC IC_{50,free} = 6.2 nM) and good in vivo pharmacodynamics in a calcium ionophore stimulated rat model after oral dosing (in vivo IC_{50,free} = 34 nM). Compound **36** mitigates the GABA binding, hepatic toxicity signal, and in vivo toxicology findings of an early lead compound **7**, with a human dose predicted to 30 mg q.d.

Introduction

While inhaled corticosteroids are the mainstay controller treatment for asthma, many patients remain uncontrolled even on escalation to high dose inhaled corticosteroids^{1,2}. Opportunities exist for new therapies which could be targeted towards these patients, and fill a much-needed therapeutic niche between inhaled steroids and progression to oral steroids or parenteral antibody therapies³. Many studies demonstrate that in patients on high dose inhaled or even oral steroids, the arachidonic acid cascade remains highly activated^{4,5}, and new treatments targeting key points on the arachidonic acid cascade could provide benefit to asthmatics with a highly activated arachidonic acid cascade, despite current medication.

The arachidonic acid cascade forms a plethora of proinflammatory and pro-resolution lipid metabolites including prostaglandins, thromboxanes and leukotrienes involved in inflammation, bronchoconstriction and pain⁶. In particular, the cysteinyl leukotrienes are strongly associated with asthma⁷, as demonstrated by the clinical efficacy of cysteinyl leukotriene modifier drugs such as the cysteinyl leukotriene receptor 1 antagonists, montelukast^{8,9} **1**, zafirlukast¹⁰ and pranlukast¹¹, and the 5-lipoxygenase inhibitor zileuton¹² **2**. While these leukotriene modulators have achieved clinical utility, they have not succeeded in becoming central to the treatment pathway in the global asthma treatment guidelines¹³ but remain an option. With such a complex cascade, it remains an open question whether the optimum point to intervene has been found with the current drugs.

The cysteinyl leukotrienes (LTC₄, LTD₄ and LTE₄) are formed from arachidonic acid in several steps, Figure 1. The enzymes 5-lipoxygenase (5-LO) and 5-lipoxygenase activating protein (FLAP) catalyze the formation of leukotriene A₄ (LTA₄) from arachidonic acid, which is converted into LTC₄ through the conjugation with glutathione by the enzyme LTC₄S^{14, 15, 16, 17}.

LTC₄ is exported from cells and is rapidly metabolized into LTD₄ by a gamma-glutamyl-transpeptidase and LTD₄ into LTE₄ by dipeptidases¹⁸. Instead of conjugation to glutathione, the reactive epoxide of LTA₄ can also be opened by the enzyme LTA₄H to generate LTB₄.

Cysteinyl leukotrienes LTC₄ and LTD₄ bind with high affinity to specific receptors (cysteinyl leukotriene receptor 2 [CYSLTR2] and cysteinyl leukotriene receptor 1 [CYSLTR1] respectively) on various target cells (leukocytes, macrophages, airway and vascular smooth muscle, endothelium, brain, adrenal gland, cardiac Purkinje cells), and elicit their well-recognized actions of anaphylaxis, smooth-muscle constriction and vascular edema by increasing post-capillary venule endothelial cell permeability and inflammation. While much focus has been given to selective inhibition of CYSLTR1 by drugs such as montelukast **1**, dual inhibition of CYSLTR1 and CYSLTR2, blocking the effects of LTC₄ and LTD₄, has been investigated clinically by the dual CysLTR1/2 antagonist gemilukast¹⁹ (Ono-6950). So far in clinical studies, dual inhibition has not shown increased efficacy over selective inhibition of CYSLTR1²⁰. While LTE₄ can elicit bronchoconstriction through CYSLTR1²¹, evidence indicates that LTE₄ can also act independently of CYSLTR1 and CYSLTR2²². Although its receptor has yet to be identified, LTE₄ has been directly implicated in driving eosinophilia²³, bronchial hyperreactivity²⁴, edema²⁵ and mucus production²⁶ in asthmatics. It has been suggested that the LTE₄ may act through the G-protein coupled receptor 99 (GPR99)^{27,28}. Cysteinyl leukotriene antagonists such as montelukast **1** are thought to primarily block the effects of the cysteinyl leukotrienes acting through CYSLTR1, due to their high affinity for CYSLTR1 and selectivity over CYSLTR2. It is unknown if they can block the effects of LTE₄ mediated through its own receptor.

Arachidonic acid can also be metabolized by 15-lipoxygenase-1, present at high levels in airway epithelial cells, eosinophils, alveolar macrophages dendritic cells and reticulocytes, to 14,15

leukotriene A4 (EXA4)²⁹. LTC4S can conjugate glutathione to this 14,15-epoxide of arachidonic acid to generate eoxin C4 (EXC4), the 14,15-regioisomer of LTC4²⁹. EXC4 is similarly exported from cells and can further be metabolized to eoxin D4 (EXD4) and eoxin E4 (EXE4). It is emerging that EXC4/D4/E4, have their own proinflammatory roles³⁰, and evidence is increasing that eoxins are raised in subpopulations of asthma including aspirin intolerant asthma³¹ and childhood asthma³².

EXA4, from oxidation of arachidonic acid by 15-lipoxygenase can also be further oxidized by 5-lipoxygenase to generate lipoxin A4 (LXA-4), one of the leukotrienes most strongly linked to resolution of inflammation³³. LXA4 is recognized as a pro-resolving mediator through its actions in inhibition of leukocyte recruitment and activation including neutrophils and eosinophils^{34, 35,36} and in restoration of injured airway epithelium by blocking the release of pro-inflammatory cytokines³⁷. Lipoxin A4 has been shown to be depressed in severe asthma³⁸.

Zileuton as a 5-lipoxygenase inhibitor can inhibit the synthesis of the cysteinyl leukotrienes, and therefore block their pharmacology irrespective of which receptor they act through. Even though at clinically approved doses zileuton **2** only partially inhibits the synthesis of the cysteinyl leukotrienes^{39,40} some clinical evidence suggests that zileuton **2** does indeed have superior efficacy to montelukast **1** in acute asthma^{41, 42}. However, zileuton's **2** dosing regimen⁴³, and requirements for liver transaminase monitoring have limited its clinical use⁴⁴. More effective inhibition of the synthesis of the cysteinyl leukotrienes has been achieved with improved 5-LO inhibitors and FLAP inhibitors which have entered clinical development.⁴⁵ Surprisingly, in clinical trials, improved inhibition of FLAP/5-LO has not led to increased efficacy⁴⁶. FLAP/5-LO inhibitors also block the production of LTB4. While LTB4 acts as a neutrophil chemoattractant,

the role of the neutrophil in asthma remains unclear. Neutrophils may drive pathology in certain asthma subtypes⁴⁷, but the lack of clinical efficacy observed with a number of neutrophil targeted drugs including LTA4H inhibitors⁴⁸ and CXCR2 inhibitors⁴⁹ may question the neutrophils pathogenic role in asthma. Neutrophils also have a protective role against bacterial colonization in the asthmatic lung⁵⁰. Hence completely inhibiting LTB4 formation may be undesirable. Furthermore, FLAP/5-LO inhibitors also inhibit the production of pro-resolution lipids such as lipoxin A4 (LXA4).

The 5-lipoxygenase inhibitor zileuton has also been shown not to inhibit the production of EXA4, so 5-lipoxygenase inhibition will not block the pathways to the eoxins⁵¹. Hence more complete inhibition of FLAP/5-LO, which may change the balance of inhibition of downstream pro inflammatory and pro-resolution pathways, may be undesirable.

With the contribution of all three cysteinyl leukotrienes to the pathology of asthma, the equivocal role of LTB4, the importance of LXA4 to resolution of inflammation, and the emerging role of the proinflammatory eoxins, we proposed that inhibiting the enzyme LTC4S may be a differentiated point to block the leukotriene cascade (Figure 1), as it would block the synthesis of LTC4, LTD4, LTE4 and EXC4, EXD4 and EXE4, while preserving LTB4 and pro-resolution LXA4.

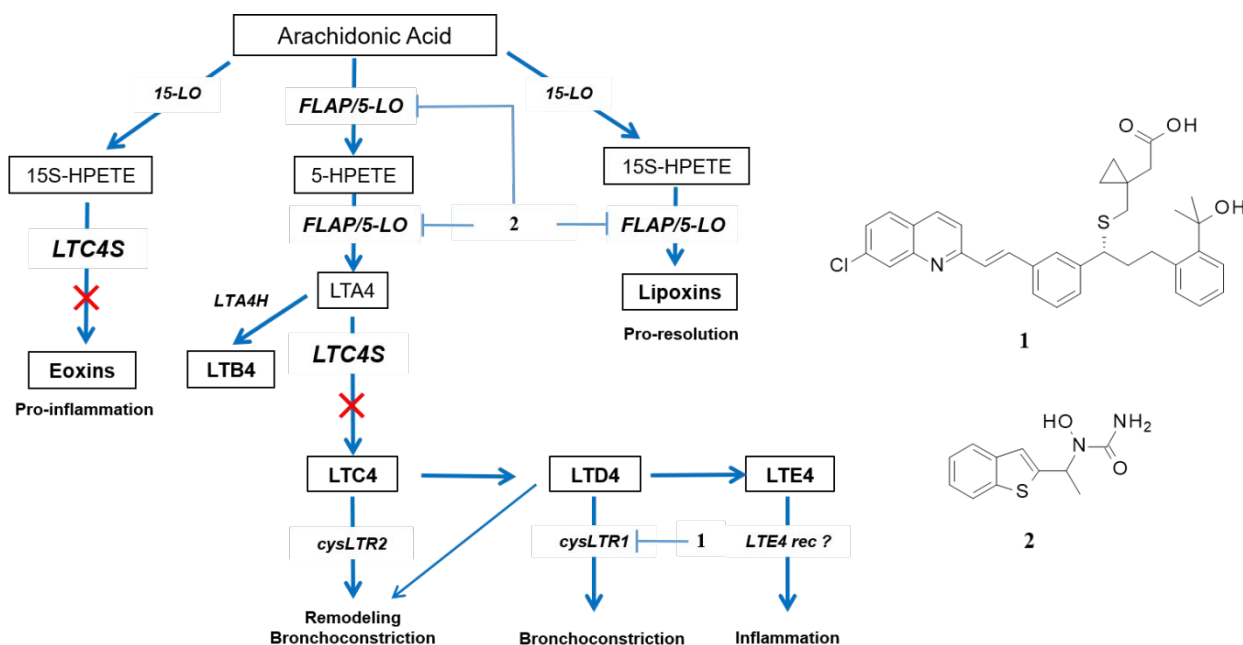


Figure 1. The differentiation in the arachidonic acid cascade of an LTC4S inhibitor (marked with a red X) compared to a cysteinyl leukotriene receptor 1 antagonist, montelukast **1** and a 5-lipoxygenase inhibitor zileuton **2**.

Therefore, we embarked on a campaign to discover potent LTC4S inhibitors which could test the clinical hypothesis that inhibiting LTC4S is a preferred point of intervention in the leukotriene cascade.

Results and Discussion

Di-benzoic acid **3** (Figure 2) was identified as an $IC_{50} = 1.9 \mu M$ hit in a focused screen of 400 commercially available compounds in the LTC4S enzyme assay. However, compound **3** suffered from poor physicochemical properties and did not inhibit cysteinyl leukotriene production in the zymosan stimulated peripheral blood mononuclear cell (PBMC) assay. Rational design focused on increasing potency and drug like properties of **3**, resulted in a mono-benzoic acid series

exemplified by compound **4**⁵². Compound **4** was potent in the LTC₄S enzyme assay (IC_{50} = 35 nM). By adding a methoxy substituent to the core pyridyl ring **5** and extending the alkyl chain on the amine to a methyl cyclopropyl, the large 2-methoxy benzamide side chain could be removed keeping potency in the LTC₄S enzyme assay (IC_{50} = 27.4 nM), improving physiochemical properties, and achieving activity in the PBMC cell assay (IC_{50} = 46.7 nM).

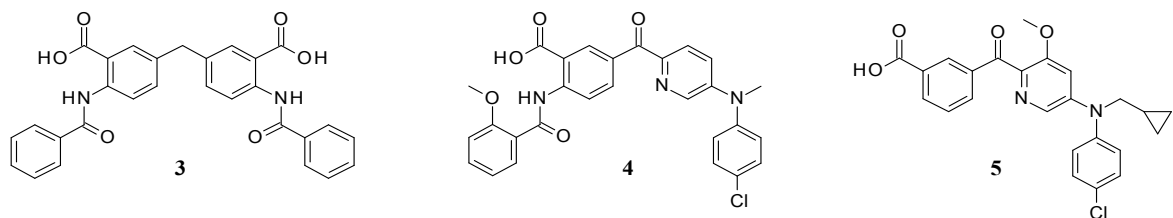
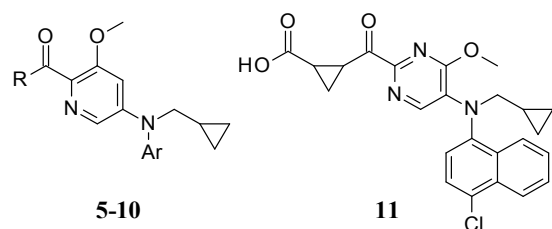


Figure 2. Original hit di-benzoic acid **3** and mono-benzoic compound **4**, and lead compound **5**.

Table 1. Initial SAR exploration

Compound	R	Ar	LTC4S ^{a,b} IC ₅₀ (nM)	HuPBMCC ^c IC ₅₀ (nM)	LogD	huHep ^d Clint (μl/min/ 10 ⁶ cells)
5	3-benzoic acid	4-chlorophenyl	27.4	46.7	1.7	28
6	2-cyclopropanecarboxylic acid	4-chlorophenyl	24.3	58.0	1.3	1.8
7	2-(1 <i>S</i> ,2 <i>S</i>)- cyclopropanecarboxylic acid	4-chloronaphthyl	1.41	9.41	2.4	5.0
8	2-(1 <i>S</i> ,2 <i>S</i>)- cyclopropanecarboxylic acid	3,4- dichlorophenyl	4.94	27.7	1.6	4.3
9	2-(1 <i>S</i> ,2 <i>S</i>)- cyclopropanecarboxylic acid	phenyl	158	1640	0.65	3.0
10	4-butanoic acid	4-chloronaphthyl	109	ND ^e	2.8	6.8
11	2-cyclopropanecarboxylic acid	4-chloronaphthyl	15.5	ND ^e	2.9	15

^aInhibitory effect of compounds on LTC₄ methyl ester production in presence of glutathione.

^bValues are mean of at least two experiments with two replicates each, unless otherwise noted.

95% confidence interval of IC₅₀ values was +/-1.7 fold ^cInhibitory effect of compounds on LTC₄

production in peripheral blood mononuclear cells (PBMCs) activated with zymosan. ^dMetabolic

stability of compound in human hepatocytes measured as disappearance of compound over time

in presence of human hepatocytes in suspension. ^eND - not determined.

Having established cell potency, the focus was to further improve properties for oral

administration. Exchanging the benzoic acid to a cyclopropane carboxylic acid **6** further lowered

lipophilicity to 1.3 while retaining potency. The low lipophilicity of **6** suggested that a more

lipophilic substituent on nitrogen would be tolerated and 4-chloronaphthyl was introduced to give

two separated enantiomers. The *S,S* enantiomer **7** was the most active enantiomer with further improved potency both in the enzyme assay $IC_{50} = 1.41$ nM and in the PBMC assay $IC_{50} = 9.41$ nM. Introducing more lipophilicity by adding an extra chlorine atom to the phenyl ring **8** also increased potency but not to the same extent as the naphthyl. Unsubstituted phenyl **9** led to a large drop in potency. Opening the cyclopropyl to the 5-oxopentanoic acid **10** gave a 78-fold drop in potency compared to **7**.

To support the design towards improved metabolic stability, metabolite identification studies in human hepatocytes were performed on selected compounds. This revealed that reduction of the ketone was a key site of metabolism. The pyrimidine analog **11** with the methoxy group shifted one position away from the keto group showed increased keto reduction and human hepatocyte Clint ($15 \mu\text{l}/\text{min}/10^6$ cells) compared to **7** having the methoxy in ortho position (Clint $5.0 \mu\text{l}/\text{min}/10^6$ cells). The cyclopropyl and the ortho-methoxy on the flanking pyridyl group thus appeared to protect the keto function from reduction, minimizing the overall hepatocyte Clint.

The high potency of compound **7** together with its reasonable stability in human hepatocytes, low percentage of keto reduction, excellent solubility ($576 \mu\text{M}$) and permeability (Caco-2 apical-basal permeability at pH6.5 105×10^6 cm/s) prompted us to profile it further. In vivo rat pharmacokinetics (Table 4) for compound **7** showed moderate clearance ($12 \text{ ml}/\text{min}/\text{kg}$) and a high volume of distribution of $2.8 \text{ L}/\text{kg}$, which is unusual for an acid⁵³, yielding an effective half-life of 2.7 hours. The rat oral bioavailability was 46%. The crossover of potency from human to rat was excellent and **7** showed a good pharmacokinetic-pharmacodynamic (PKPD) profile in a calcium ionophore challenge rat model (Figure 3). Furthermore, compound **7** had no significant cytochrome P450 inhibition ($>20 \mu\text{M}$ for CYP1A2, CYP2C9, CYP2C19, CYP3A4 and CYP2D6), no hERG inhibition and was negative in the 5 strain Ames genotoxicity assays⁵⁴.

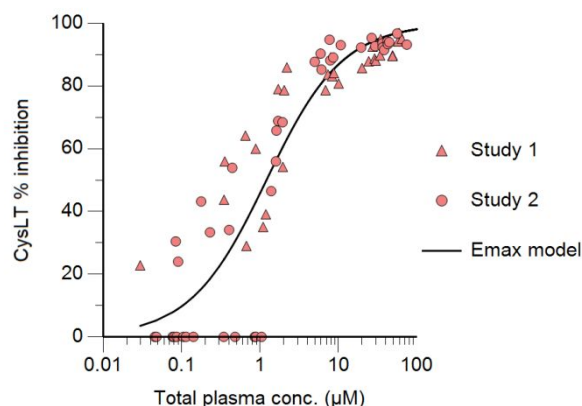


Figure 3 Effect of **7** on inhibition of CysLT production in the rat calcium ionophore challenge model. Compound **7** was administered orally two hours prior to a calcium ionophore challenge with A23187 via terminal bronchoalveolar lavage (BAL), and plasma concentration of **7** was determined at time of PD (CysLT in BAL fluid) measurement. Data from two consecutive experiments (1-90 mg/kg in study 1, triangles; 0.1-90 mg/kg in study 2, circles) were pooled and fitted to a sigmoid Emax model, yielding an EC_{50} of 1.21 μ M (CI 95% 0.89-1.5).

To further evaluate **7** the compound was progressed to a rat 7-day oral toxicology study at 30 mg/kg and 300 mg/kg (6 animals per group including controls 3 males + 3 females) and was well tolerated at both doses. A complete histochemical assessment was undertaken and unilateral sperm granulomas in the epididymal sperm duct were found in the high dose male group of rats. These findings could potentially be attributed to potent inhibition of organic anion transporting polypeptide 1B1 (OATP1B1) (0.6 μ M) and/or other ATP-binding cassette (ABC) transporters, as these have been shown to protect sperm cells from xenobiotics.⁵⁵ According at the exposures

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3 achieved in the safety study, approaching 140 μM ($\sim 1 \mu\text{M}$ free) at C_{max} , OATP1B1 would have
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5 been inhibited.
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8 In a secondary pharmacology assessment in a panel of >150 in vitro radio ligand binding and
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10 enzyme assays covering a diverse set of enzymes, receptors, ion channels, and transporters it was
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12 found that **7** overall had a clean profile but was a potent GABA gated Cl^- channel⁵⁶ (rat cerebral
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14 cortex) binder with IC_{50} 0.074 μM . GABA agonists are anxiolytic, anticonvulsant,
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16 antihyperalgesic, can cause sedation, would impair motor function, cause muscle relaxation,
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18 ataxia, dizziness, depression, anterograde amnesia and antagonists could be excitatory, pro-
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20 convulsant and lead to seizures⁵⁷.
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25 Due to the moderate metabolic stability, the transporter interactions, the GABA binding and the
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27 testicular toxicity findings in rat, compound **7** was not progressed further. A further optimisation
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29 campaign was launched targeting high PBMC potency and low hepatocyte turnover together with
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31 a low logD and high ligand lipophilic efficiency to reduce a general logD driven toxicology risk,
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33 including the interactions with transporter proteins and GABA binding.
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37 A screening cascade to mitigate the deficiencies of compound **7** was established, and assays
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39 conducted in parallel defined as “waves”. Wave 1 contained the critical assays guiding compound
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41 design including, LTC4S enzyme and PBMC cell potencies, physicochemical properties,
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43 metabolic stability, as well as GABA binding. For compounds progressing to wave 2,
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45 permeability and rat pharmacokinetics were included to explore in vivo pharmacokinetic
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47 properties and in vitro to in vivo scaling of clearance. Finally, compounds were assessed for dog
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49 pharmacokinetics as a second pharmacokinetic species to provide additional confidence in
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51 predicting human pharmacokinetics. They were also assessed for efficacy in the rat calcium
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53 ionophore challenge PKPD model, in which the degree of inhibition of cysteinyl leukotriene
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production measured in rat bronchoalveolar lavage after calcium ionophore challenge was measured after oral administration of the LTC₄S inhibitor. For shortlisting of potential clinical candidates, an integrated in vitro approach to risk assess the potential for liver toxicity and idiosyncratic drug reactions in humans was undertaken⁵⁸. This consisted of assays addressing 1) covalent binding of parent compound or metabolites to human hepatocytes, 2) toxicity to transformed human liver epithelial 2 (THLE) cells with and without metabolic activation, 3) mitochondrial toxicity in HepG2 cells, and 4) inhibition potential against human bile salt export pump (BSEP) and multidrug resistance associated protein 2 (Mrp2). Additional safety liabilities were assessed through Ames assays, and pharmaceutical developability was assessed through crystallinity and melting point.

An apo structure of human LTC₄S (2IIU), and a structure in complex with glutathione (2UUH) exist in the literature⁵⁹. To support structural based design, we determined the structure of compound **12**⁶⁰ (LTC₄S IC₅₀ = 2.4 nM) bound to LTC₄S (Figure 4). The crystal structure revealed the binding mode of **12** in the substrate pocket of LTC₄S and suggested that the activity resides in the *S,S* enantiomer (Supporting Figure S3). However, the data cannot unambiguously define the stereochemistry of the bound compound. The acidic side chain points towards the glutathione binding site and forms hydrogen bonds to the side chain of Arg104 and the phenol oxygen on Tyr93. Furthermore, the side chain of Arg90 was arranged to coordinate the keto-group and one of the pyrimidine nitrogens (Figure 4A). Tyr59 π -stacked with the pyrimidine ring (Figure 4B) The methoxy group and the naphthalene ring reside in enclosed cavities while the *N*-cyclopropyl methyl side chain faces the entrance of the binding pocket (Figure 4A). Compound *S,S*-**12** takes advantage of the polar groups available to it while matching the shape of the binding pocket well. The structure also suggested that the naphthyl pocket would allow for a small

substituent of the size of fluorine, chlorine or methyl in the 2-position and the *N*-cyclopropyl methyl was expected to tolerate modifications as it pointed out of the binding pocket.

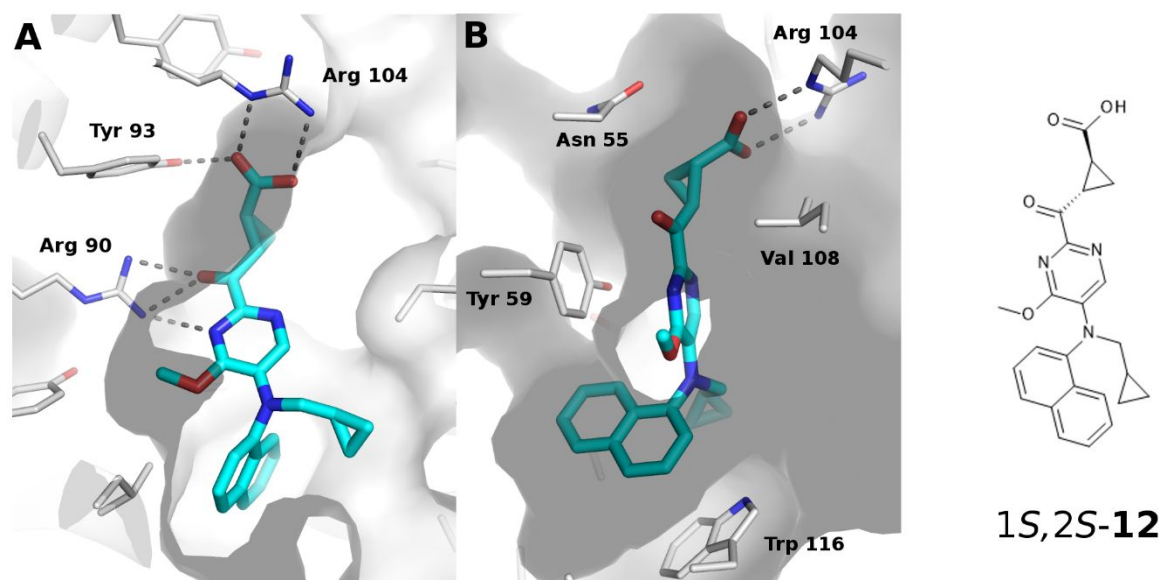
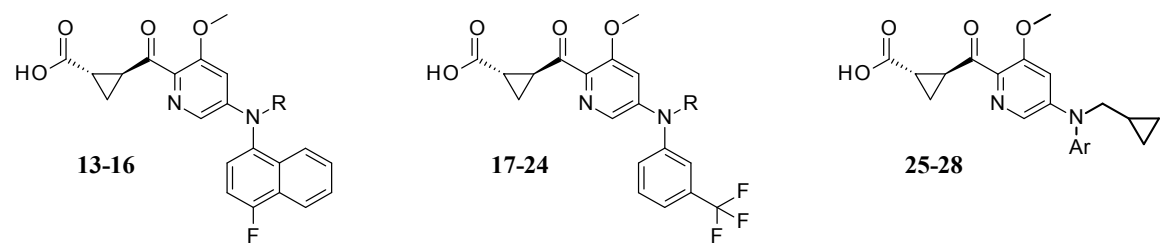


Figure 4. X-ray crystal structure of 1S,2S-12 complexed with human LTC₄S (pdb 6R7D). (A) The acid forms hydrogen bonds to the side chain of Arg104 and the phenol oxygen on Tyr93. The side chain of Arg90 is arranged to coordinate the keto-group and one of the pyrimidine nitrogens. *N*-cyclopropyl methyl side chain faces the entrance of the binding pocket (B) Tyr59 π -stacked with the pyrimidine ring.

In the first attempt to reduce lipophilicity, a set of analogues to **7** were prepared with a 4-fluoronaphthyl (Table 2) instead of the 4-chloronaphthyl and with variable alkyl side chains (cyclopropylmethyl **13**, 2-methylpropyl **14**, propyl **15** and ethyl **16**). This revealed that the human hepatocyte intrinsic clearance was lowered by changing the *N*-alkyl side chain from 2-methylpropyl as in **14** (56.4 $\mu\text{l/min}/10^6\text{cells}$) to a *n*-propyl **15** (7.57 $\mu\text{l/min}/10^6\text{cells}$) and to ethyl **16** (3.8 $\mu\text{l/min}/10^6\text{cells}$) following the reduction in lipophilicity. Acceptable cellular PBMC

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3 potency (17 nM) was achieved also with compound **16** with the lowest logD in the comparison.
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5 Reducing logD also had a clear effect on the GABA binding potency, that was reduced from IC₅₀
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7 = 0.64 μM for compound **13** to IC₅₀ = 2.9 μM for compound **16**. In a reactive metabolite study of
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9 compound **13** with added glutathione (GSH) to trap formed electrophilic species, GSH adducts
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11 were found. However, in this series of compounds no GSH adducts could be identified for
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14 compounds **14-16**.
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Table 2. Optimization of *N*-alkyl and *N*-aryl groups. Reducing GABA binding and formation of glutathione adducts.



Compound	R or Ar	LTC4S ^{a,b} IC ₅₀ (nM)	HuPBMC ^c IC ₅₀ (nM)	LogD	GSH adducts ^d	GABA ^e (μM)	huHep Clint ^f (μl/min/10 ⁶ cells)
13	cyclopropylmethyl	0.758	26.5	1.9	Yes	0.64	5.4
14	2-methylpropyl	0.381	2.26	2.2	No	ND ^g	56
15	propyl	0.626	7.09	1.8	No	3.5	7.6
16	ethyl	1.05	16.8	1.4	No	2.9	3.8
17	cyclopropylmethyl	6.42	52.4	1.4	No	11	2.3
18	2-methylpropyl	4.74	20.6	1.6	ND ^g	ND ^g	2.9
19	2-hydroxy-2-methylpropyl	31.8	ND ^g	0.0	ND ^g	>100	ND ^g
20	2-fluoro-2-methylpropyl	8.65	55.5	0.9	No	54	<1.5
21	propyl	7.90	58.4	1.2	ND ^g	19	7.2
22	ethyl	13.0	113	0.7	ND ^g	20	3.1
23	2,2,2-trifluoroethyl	11.5	156	1.1	Yes	ND ^g	ND ^g
24	4-fluorobenzyl	13.5	157	1.9	No	ND ^g	4.2
25	2-fluoro-5-(trifluoromethyl)phenyl	2.08	14.0	1.2	No	8.2	<1.1
26	5-chloro-2-fluorophenyl	3.71	34.3	1.1	No	ND ^g	1.3
27	3-chloro-4-fluorophenyl	5.26	42.3	1.2	ND ^g	3.0	3.1
28	5-chloro-2,4-difluorophenyl	1.97	27.6	1.1	No	5.4	<1.5

^aInhibitory effect of compounds on LTC₄ methyl ester production in presence of glutathione.

^bValues as mean of at least two experiments with two replicates each unless otherwise noted.

95% confidence interval of IC₅₀ values was +/-1.7-fold. ^cInhibitory effect of compounds on LTC₄

production in peripheral blood mononuclear cells (PBMCs) activated with zymosan. ^dCompounds were incubated in human liver microsomes in the presence glutathione and NADPH at pH 7.4 and 37°C. ^eBinding to GABA gated Cl⁻ channel⁵⁶ (rat cerebral cortex) ^fMetabolic stability of compound in human hepatocytes measured as disappearance of compound over time in presence of human hepatocytes in suspension. ^gND - not determined

Next, to further reduce the lipophilicity we replaced the 4-fluoronaphthyl with 3-(trifluoromethyl)phenyl. On that scaffold the *N*-alkyl group was optimized with the primary aim to afford low human hepatocyte Clint. The *N*-cyclopropyl methyl derivative **17** with logD_{7.4} of 1.4 was synthesized as a reference for the *N*-alkyl optimization and displayed a GABA binding of 11 μM and no GSH adducts. It displayed a LTC4S potency of IC₅₀ = 6.42 nM and a lower human hepatocyte Clint (2.3 μl/min/10⁶cells) as compared to the matched pair **13** (5.4 μl/min/10⁶cells). Further, compared to **7**, compound **17** afforded only a 5-fold reduced LTC4S potency accompanied with a 10-fold decrease of lipophilicity. Changing to *N*-2-methylpropyl (**18**) gave a compound with similar LTC4S potency (IC₅₀ = 4.74 compared to 6.42 nM) but with two-fold increased PBMC potency (IC₅₀ = 20.6 compared to 52.4 nM).

Similar to the structure activity relationship for the 4-fluoronaphthyls **13-16**, decreasing the size of the R-group from 2-methylpropyl to propyl and ethyl lead to a 5-fold decrease of the PBMC potency. The 3-(trifluoromethyl)phenylamine **18** bearing a *N*-2-methylpropyl displayed a reduced human hepatocyte Clint compared to the matched pair fluoro naphthyl **14**, 2.9 and 56 μl/min/10⁶ cells respectively.

Hydroxylation **19** and fluorination **20** of the 2-methylpropyl led to decreased LTC₄S potency (IC_{50} = 32 and 8.65 nM respectively) and the human hepatocyte Clint was reduced to below the detection limit (<1.5 μ l/min/ 10^6 cells) for compound **20**. Exchanging *N*-ethyl (**22**) to trifluoroethyl (**23**) had no effect on the LTC₄S binding (13.0 versus 11.5 nM), and increasing the size of the R-group to *N*-4-fluoro benzyl (**24**) did not contribute to an improvement in potency (IC_{50} = 13.5 nM).

In parallel to exploring the best option for the *N*-alkyl group, the phenyl substitution pattern was explored (Table 2). Modelling suggested that the 6-position of the phenyl ring may tolerate a small substituent such as a fluorine. Compound **25** displayed an improved potency in both the LTC₄S binding assay and in the PBMC assay together with a reduced logD compared to the des-fluoro matched pair **17**. By changing the 3-trifluoromethyl substituent in **25** to a 3-chloro substituent **26** the low logD was retained together with a slight decrease in potency. Moving the fluorine substituent to the 4-position **27** resulted in a further decrease in potency and an increase of the logD. However, combining the 4- and 6-fluoro substituent as in **28** led to an increase in LTC₄S binding and PBMC potency IC_{50} = 1.97 and IC_{50} = 28 nM respectively, together with reduction of human hepatocyte Clint <1.5 μ l/min/ 10^6 cells similar to **25**.

In conclusion *N*-2-methylpropyl **18** and *N*-2-fluoro-2-methylpropyl **20** were identified as the preferred *N*-alkyl substituents in combination with the 3-trifluoromethyl phenyl due to the favourable PBMC potency and human hepatocyte Clint profiles. Of significance is the low logD (0.9) for **20** where the fluorine-carbon bond dipole reduces the lipophilicity approximately 7-fold compared to *N*-2-methylpropyl, leaving flexibility for further optimisation. The 5-chloro-2,4-difluorophenyl and the 2-fluoro-5-(trifluoromethyl)phenyl represented the two most optimal substituted phenyls identified.

The preferred *N*-aryl and *N*-alkyl groups identified above (Table 2) were combined to give pyridines **29-32** (Table 3). The *N*-2-methylpropyls **29** and **30** were more potent in the PBMC than the *N*-2-fluoro-2-methylpropyls **31** and **32**, $IC_{50} = 6.8$ and $IC_{50} = 8.5$ nM compared to $IC_{50} = 31$ and $IC_{50} = 16$ nM. However, both had higher logD and displayed a higher human hepatocyte Clint (3.9 and 4.9 $\mu\text{L}/\text{min}/10^6\text{cells}$) than the matched pairs (<1.0 and <1.7 $\mu\text{L}/\text{min}/10^6\text{cells}$).

From the X-ray crystal structure of **12** complexed with human LTC4S (Figure 4B) a π -stacking interaction between the central pyridyl with Tyr59 was identified. We hypothesized that the affinity to the central core could be improved by the introduction of alternative electron poor aryls. Quantum Mechanical (QM) calculations on a representative system (Figure 5) were performed with a number of selected core replacements. Relative energies are given between the reference core ring from **12** (**C-Ref**), whereas higher energies suggest a more favorable interaction. The high relative solvation energy consistent with the relative gas phase energy suggested that the pyridine core ring **C-E** could be replaced by pyrazine **C-F**, to improve affinity to LTC4S.

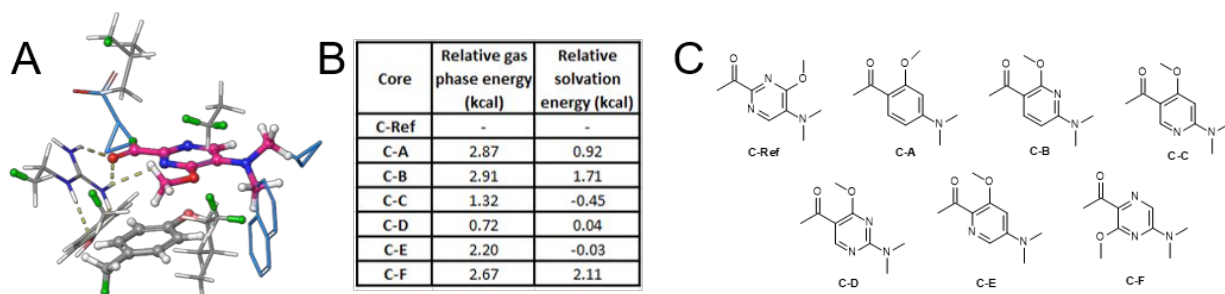
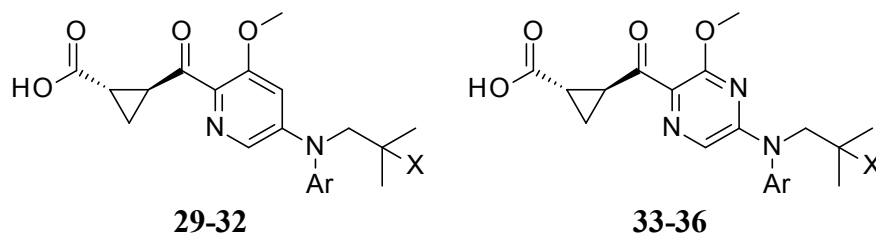


Figure 5. (A) Reduced system for QM calculations with the core ring (magenta) of ligand **12** (blue) with selected amino acids (grey) based on the X-ray of **12**. Atoms fixed during the geometry optimization are colored green. (B) Relative energies are given between the reference core ring from **12** (C Ref) and the modelled variants. (C) Core ring models used in the QM calculations.

Combining the pyrazine core C-F with the same preferred *N*-aryls and *N*-alkyls as above gave compounds **33-36**. As predicted the pyrazine core displayed a 2-fold increase in the LTC4S binding and PBMC potency compared to the pyridine matched pairs. Again, the *N*-2-fluoro-2-methylpropyls **35** and **36** display low human hepatocyte Clint, 1.5 and <1.2 $\mu\text{L}/\text{min}/10^6\text{cells}$, respectively. Due to the lower in human hepatocyte Clint of **36** relative to **35**, **36** was chosen for progression.

Table 3. Combining optimal phenyl and *N*-alkyl groups with either pyridyl or pyrazine central aryl.



Compound	X	Ar	LTC4S ^{a,b} IC ₅₀ (nM)	HuPBMC ^c IC ₅₀ (nM)	LogD	huHep Clint ^d (μl/min/10 ⁶ cells)
29	H	2-fluoro-5-(trifluoromethyl)phenyl	0.66	6.8	1.4	3.9
30	H	5-chloro-2,4-difluorophenyl	0.70	8.5	1.3	4.9
31	F	2-fluoro-5-(trifluoromethyl)phenyl	0.63	31	0.8	<1.0
32	F	5-chloro-2,4-difluorophenyl	0.60	16	0.8	<1.7
33	H	2-fluoro-5-(trifluoromethyl)phenyl	0.36	3.5	2.0	2.9
34	H	5-chloro-2,4-difluorophenyl	0.28	4.1	1.8	3.0
35	F	2-fluoro-5-(trifluoromethyl)phenyl	0.25	7.8	1.2	1.5
36	F	5-chloro-2,4-difluorophenyl	0.28	10	1.1	<1.2

^aInhibitory effect of compounds on LTC₄ methyl ester production in presence of glutathione.

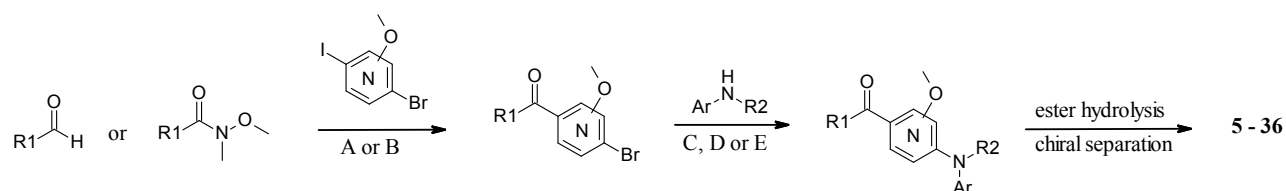
^bValues as mean of at least two experiments with two replicates each unless otherwise noted.

95% confidence interval of IC₅₀ values was +/-1.7-fold ^cInhibitory effect of compounds on LTC₄ production in peripheral blood mononuclear cells (PBMCs) activated with zymosan. ^dMetabolic stability of compound in human hepatocytes measured as disappearance of compound over time in presence of human hepatocytes in suspension.

Chemistry. Compound **36** and analogues described above were derived from three main building blocks by the general methods depicted in Scheme 1. First the carboxylic chain was introduced

on the corresponding iodo bromo substituted heterocycle through a Grignard reaction on either the Weinreb amide (method A) or the aldehyde followed by oxidation to the ketone (method B). The formed heterocyclic bromide was further coupled to secondary amines (method C) or primary amines followed by alkylation (method D) under Buchwald conditions. Alternatively, in the case of 3-methoxypyrazines the secondary amine was deprotonated with a base and added through nucleophilic aromatic substitution to the electron poor 3-methoxypyrazine (method E). Ester hydrolysis and chiral separation of enantiomer mixtures then gave the final products. Non-commercial secondary amines used in method C and E were synthesized from commercial primary amines by acylation with acyl halides followed by reduction with borane.

Scheme 1. General method for preparation of compounds **5-36**^a

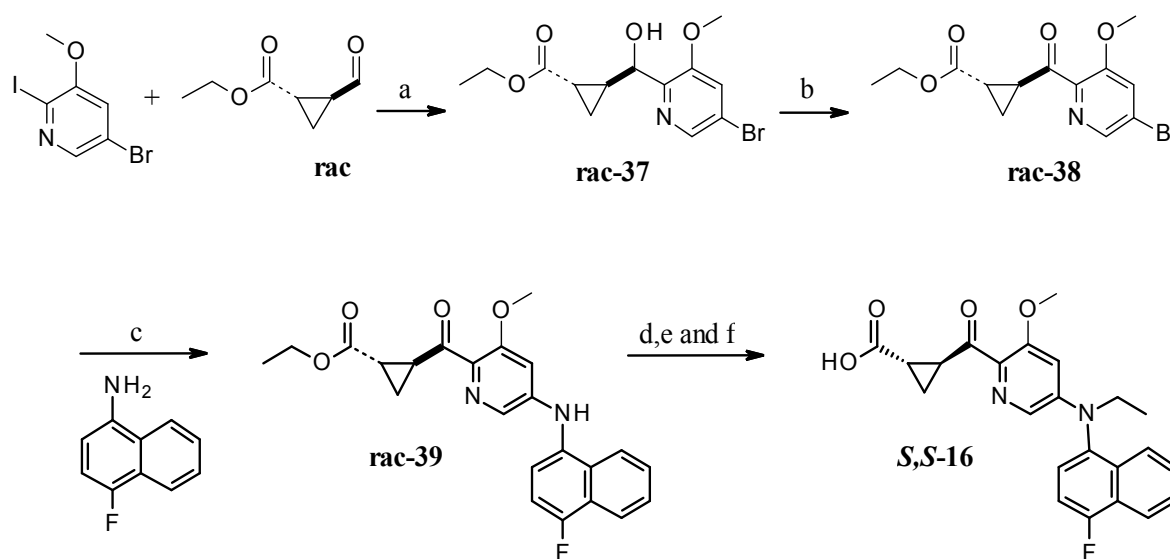


^aFor R1, R2 and Ar see Table 1-3 and experimental. Method A: Weinreb amide, $(\text{CH}_3)_2\text{CHMgCl} \cdot \text{LiCl}$; Method B: aldehyde, $(\text{CH}_3)_2\text{CHMgCl} \cdot \text{LiCl}$ followed by MnO_2 ; Method C: secondary amine, Pd-catalyst, ligand Cs_2CO_3 ; Method D: primary amine, Pd-catalyst, ligand, Cs_2CO_3 followed by NaH and alkyl bromide; Method E: secondary amine, $((\text{CH}_3)_3\text{Si})_2\text{NK}$.

The detailed synthesis of compound **16** and **36** is outlined below and described in the experimental part. Compound **16** (Scheme 2) was prepared according to a combination of route B and D. Trans-ethyl 2-formylcyclopropanecarboxylate was reacted with 5-bromo-2-iodo-3-methoxypyridine in the presence of iso-propyl magnesium chloride. The formed alcohol **rac-37**

was oxidized with manganese dioxide to form the ketone **rac-38** that following a palladium catalysed coupling with 4-fluoronaphthalen-1-amine gave **rac-39**. Alkylation with ethyl bromide followed by saponification with sodium hydroxide and chiral separation afforded compound **16**. For some chiral compounds a standard procedure was developed where intermediate **rac-38** was submitted to chiral purification followed by mixing the enantiomers as a 90/10 mixture of active/inactive component. This mixture was used for synthesis of test compounds where the contamination of up to 10% inactive enantiomer in the final compound was utilized to secure chiral separation of target compound enantiomers and determination of enantiomer excess (% ee).

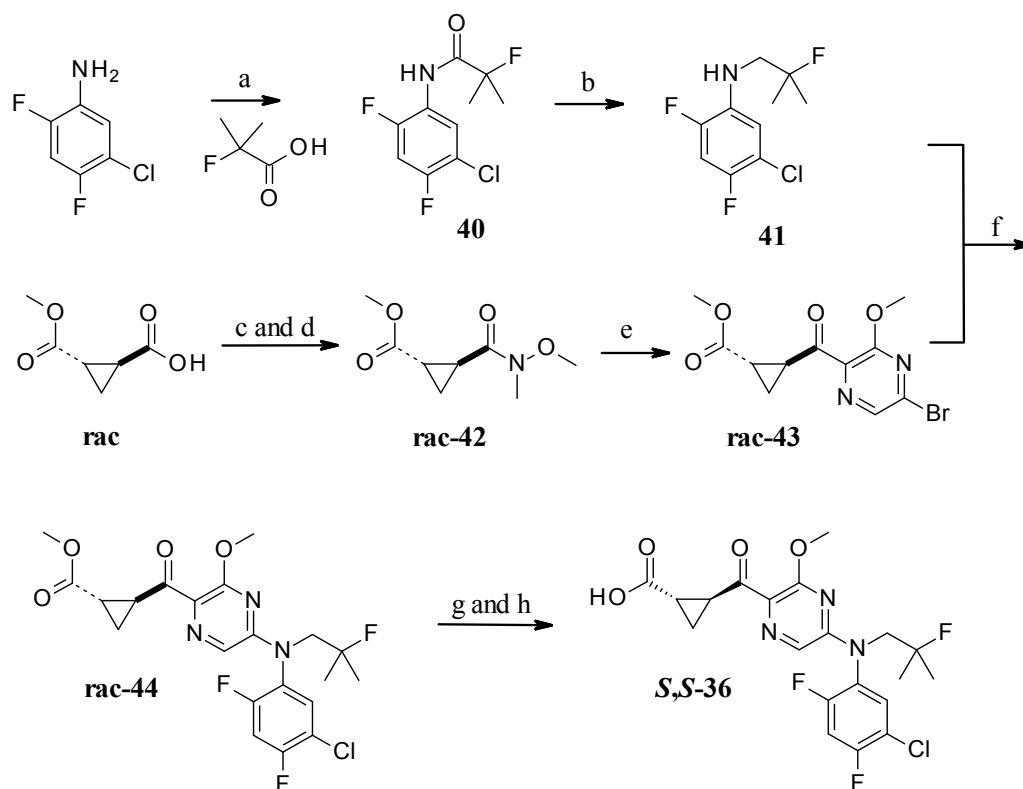
Scheme 2^a. Synthesis of **16**.



^a(a) $(\text{CH}_3)_2\text{CHMgCl} \cdot \text{LiCl}$, tetrahydrofuran; (b) MnO_2 , tetrahydrofuran; (c) $\text{Pd}(\text{OAc})_2$, racemic BINAP, Cs_2CO_3 , toluene; (d) NaH , ethyl bromide, N,N -dimethylformamide; (e) NaOH ; (f) chiral chromatography

In the case of **36** (Scheme 3), the secondary amine **41** was prepared from the 5-chloro-2,4-difluoro-aniline and 2-fluoro-2-methyl-propanoic acid with the help of isobutyl chloroformate and 4-methylmorpholine followed by reduction of the amide **40** with borane tetrahydrofuran complex. The coupling partner **rac-43** was prepared through a Weinreb–Nahm ketone⁶¹ synthesis reacting 5-bromo-2-iodo-3-methoxypyrazine with the Weinreb amide **rac-42** obtained from trans-2-methoxycarbonylcyclopropanecarboxylic acid under standard conditions. Compound **41** was deprotonated with potassium bis(trimethylsilyl)amide at -20°C and reacted with compound **rac-43** to yield **rac-44** that after saponification with sodium hydroxide and separation of the enantiomers gave compound **36**.

Scheme 3. Synthesis of **36**.^a



^a(a) isobutyl chloroformate, 4-methylmorpholine; (b) borane tetrahydrofuran complex, tetrahydrofuran, 60°C; (c) Oxalyl chloride; (d) *N,O*-dimethyl hydroxylamine hydrochloride; (e) $(\text{CH}_3)_2\text{CHMgCl}\cdot\text{LiCl}$, 5-bromo-2-iodo-3-methoxypyrazine, tetrahydrofuran; (f) $((\text{CH}_3)_3\text{Si})_2\text{NK}$, -20°C; (g) NaOH; (h) chiral chromatography

The protein crystal structure of **12** did not conclusively define the absolute stereochemistry of the potent enantiomer of the compound. Therefore, the structure and absolute stereochemistry of **36**, were generated by single crystal X-ray diffraction and confirmed the *S,S* configuration of the active enantiomer (Figure 6 and supplementary pages for detail).

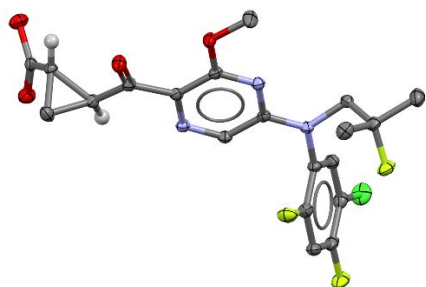


Figure 6. Single crystal X-ray diffraction structure of **36**. Thermal ellipsoids drawn at the 50% probability level. Hydrogen atoms and methanol moiety are omitted for clarity.

Preclinical pharmacokinetic profile, in vitro pharmacology and off-target safety. Pyrazine **36** was selected for progression into preclinical development. Its profile is shown in Table 4. Compound **36** showed low turnover in human, monkey, dog and rat hepatocyte incubations which was also reflected in low plasma clearance observed in vivo in the preclinical species

(<2% of liver blood flow). The unbound steady state volume of distribution (Vss) varied between rat and dog, which posed a risk for the prediction of the human volume of distribution. To increase confidence in predicting human Vss, the pharmacokinetics of **36** was explored in cynomolgus monkey, in which the unbound Vss was found to be similar to dog. As dog and monkey were more consistent, the human Vss estimate was based on these two species. Compound **36** had excellent pharmaceutical properties, being a non-hygroscopic crystalline anhydrate with a melting point of 129 °C with no other thermal events.

Table 4. Comparison of preclinical profile of compounds **7** and **36**.

	7	36
hPBMC IC _{50,free} (nM) ^a	1.1	6.2
In vivo inh of CysLT EC _{50,free} (nM) ^{a,b}	7.5	34
logD _{7.4}	2.4	1.1
Solubility pH 7.4 (μM)	576	2080
PPB (%free) hu/rat/dog/cyno	0.66/0.62/0.21/ND ^c	3.6/2.5/5.9/3.0
Caco-2 Papp (10 ⁻⁶ cm s ⁻¹) / efflux ratio	105 / ND ^c	61 / 3.2
Pharmacokinetic parameters in rat / dog / cyno^{d,e}		
t _{1/2,effective} ^f (h)	2.7 / 4.8 / ND ^c	26 / 6.1 / 12
Fg %	46 / 91 / ND ^c	100 / 100/ 90
CL ^h (ml/min/kg)	12 / 2.4 / ND ^c	2.3 / 1.8 / 0.6
V _{ss} ⁱ (L/kg)	2.8 / 1.1 / ND ^c	5.3 / 1.0 / 0.6
Safety & Selectivity		
GABA binding (μM)	0.074	>100
OATP1B1(μM)	0.6	3.2
CYP inhibition (5 isoforms)	>20μM	>30μM
GSH adducts	Yes	No

^aThe IC50 values were converted to unbound (free) values (see experimental section under respective assay); ^bInhibition of CysLT in rat calcium ionophore challenge model; ^cND - not

determined; ^dHan Wistar rats, male Beagle dogs, cynomolgous monkeys ^edose in rat 0.5 mg/kg iv; 1 mg/kg po, dose in dog 1 mg/kg iv; 2 mg/kg po; dose in cyno 1 mg/kg iv; 2 mg/kg po; $t_{1/2, \text{effective}} = \ln 2 \times V_{ss} / Cl$; ^gF oral bioavailability; ^hCL clearance; ⁱV_{ss} volume of distribution at steady state.

Compound **36** showed no reactivity to glutathione and did not inhibit cytochrome P450 isoforms up to 30 μM. It displayed excellent selectivity in a panel of >200 in vitro radio ligand binding and enzyme assays covering a diverse set of enzymes, receptors, ion channels, and transporters. The only activity identified within 100 times of the human predicted C_{max}, was an interaction with GABA-A as a positive allosteric modulator at 24 times human predicted C_{max}. A positive allosteric modulator would enhance an endogenous response to GABA. No seizures were observed in any of the rat toxicology studies, at up to 122 times the human predicted exposure, and the in vitro observation was assessed as a low risk for progression. Additionally, compound **36** had no activity below 33 μM at a panel of cardiovascular ion channels and was negative in a 5-strain Ames genetic toxicology assessment. Taken together, these data demonstrate that compound **36** is a highly selective LTC₄S inhibitor with low risk for off-target effects.

In vivo efficacy and safety. Compound **36** was evaluated head to head with the original lead **7** in a 7-day rat toxicology study. In the study, doses were chosen so **36** achieved matching or greater exposures than those achieved by **7** earlier (Table 5).

Table 5^a. Seven day rat toxicology study and results for compounds **7** and **36**.

	study design	no adverse effect level (NOAEL)	lowest adverse effect level	findings at highest dose	Safety margins ^e
7	Rat 3M+3F/group 30 & 300 mg/kg	C _{max} 47 µM AUC 200 µM.hr	C _{max} 144 µM AUC 1830 µM.hr	Sperm granuloma (moderate-severe) Bilirubinemia Liver hypertrophy	7-16 times
36	Rat 6M/group 10 & 100 mg/kg	C _{max} 349 µM AUC 4500 µM.hr	NV	No testicular observations Liver hypertrophy	200 times

^aNV no value, ^eMargins @NOAEL to human predicted total AUC

Compound **7** again showed evidence of sperm granuloma in the high dose group providing margins to a no adverse effect level of 7-16 times based on the predicted human exposures at the predicted efficacious dose. Compound **36** showed no signs of testicular toxicity, and only adaptive changes in the liver due to cytochrome P450 induction which were not judged adverse, providing a 200 fold margin between the no adverse effect level and the predicted human exposure at the predicted therapeutic dose. Hence **36** was chosen as a progressable candidate.

The PKPD relationship for in vivo LTC₄S inhibition by **36**, was explored in a calcium ionophore challenge rat model, measuring inhibition of CysLT production in the lung following oral delivery of **36**. A dose response experiment was first performed, where **36** was administered two hours prior to a terminal bronchoalveolar lavage with calcium ionophore A23187, followed by a time-course experiment, where the challenge was done 0.5, 2, 6 and 24 hours post a single dose, and 0.5, 2 and 6 hours after four days of daily oral dosing. The relationship between **36** plasma concentration and reduction of CysLT in BAL fluid (BALF) across both experiments was well described by a sigmoid E_{max} model (Figure 7), suggesting a direct relationship between inhibition of leukotriene production in the rat lung, and the instantaneous plasma level at the timepoint of the PD measurement. The EC₅₀ for inhibition of CysLT production in rat lung was estimated to

1.36 μM (CI 95% 0.99-1.7), corresponding to an unbound EC_{50} of 34 nM, which is within 5.2-fold of the free IC_{50} measured in the human PBMC assay of $\text{IC}_{50} = 6.5$ nM.

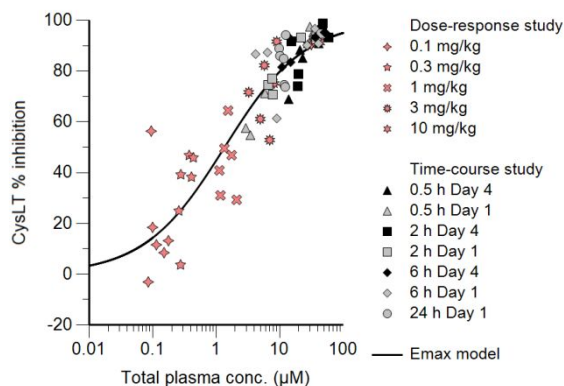


Figure 7. Effect of **36** on inhibition of CysLT production in the rat calcium ionophore challenge model. In the dose-response study **36** was administered orally two hours prior to a calcium ionophore challenge via BAL. In the time-course study, the challenge was done 0.5, 2, 6 or 24 hours post a single dose, and 0.5, 2 or 6 hours after four days of daily oral dosing at 3 or 10 mg/kg. Data across both studies were pooled and fitted to a sigmoid E_{max} model, yielding an EC_{50} of 1.36 μM (CI 95% 0.99-1.7).

Human dose predictions. The human effective dose at steady state for **36** was predicted, assuming a direct PKPD relationship in man, as was demonstrated for rat in the calcium ionophore challenge model, and the requirement to maintain inhibition of at least 75% throughout the dosing period (free plasma concentration $3 \times \text{PBMC } \text{IC}_{50, \text{free}}$)⁶². The human PK parameters used to calculate a dose aiming to achieve the corresponding plasma concentration were: a

volume of distribution of 0.6 L/kg, predicted based on the average unbound volumes observed in dog and cynomolgus monkey, and human clearance of 0.5 ml/min/kg, estimated using a liver blood flow method. The predicted human effective half-life was 14 hours, and assuming high and rapid absorption, the human predicted dose was 30 mg once a day. With the high permeability (Caco2 Papp 47×10^6 cm/s) and solubility of **36**, the maximal absorbable dose^{63,64} was calculated to be 10 g, suggesting a low risk for absorption limiting exposure at a 30 mg effective dose.

Pharmacological profile of 36. We hypothesised a LTC₄S inhibitor could provide a differentiated profile from current leukotriene modifiers by inhibiting cysteinyl leukotriene production while preserving LTB₄ and LXA₄. (Figure 1). To demonstrate this, we incubated human whole blood (HWB) from both healthy subjects (Figure 8A) and asthmatics (Figure 8B) with different concentrations of compound **36**, montelukast **1** or zileuton **2** followed by calcium ionophore stimulation to analyse the metabolite production of the activated arachidonic acid (AA) pathway. As predicted, compound **36** and zileuton **2** inhibited the formation of CysLTs, while montelukast **1** showed little effect on the production of these metabolites (Figure 8). While inhibiting CysLTs, compound **36** not only preserved LXA₄, but significantly enhanced lipoxin production without shunting the substrate LTA₄ towards LTB₄ formation. The modulation of these metabolites occurred in a dose-dependent manner. The three compounds showed a similar regulation of the pathway in healthy blood versus asthmatic blood in vitro (Figure 8).

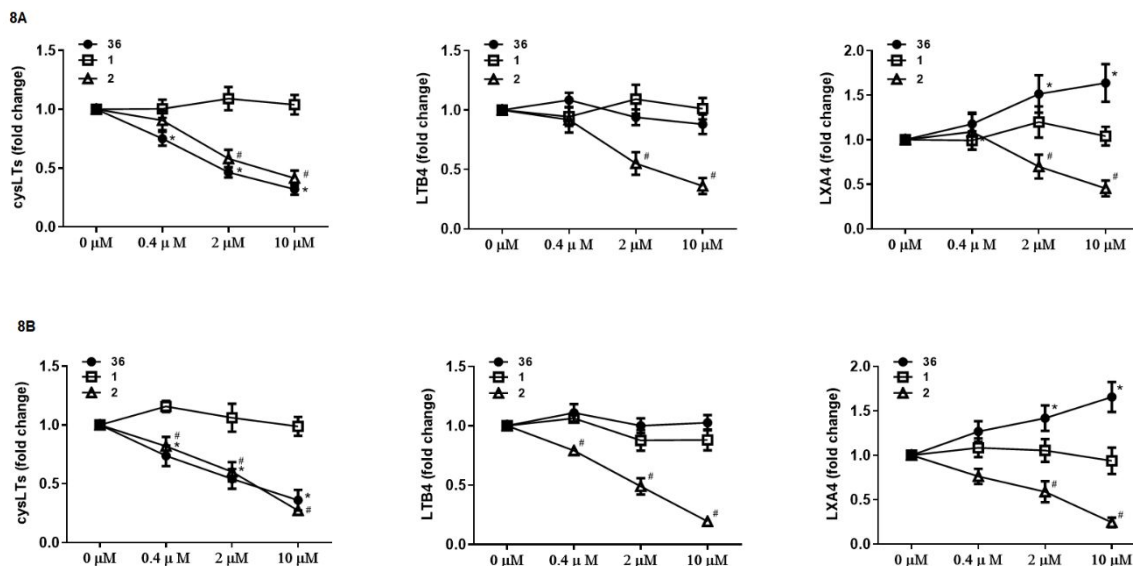


Figure 8. Compound **36** regulates the arachidonic acid (AA) pathway differently from montelukast **1** and zileuton **2** in human whole blood. Metabolite productions stimulated by calcium ionophore in the absence or presence of different concentrations of **36**, montelukast **1** or zileuton **2** were analysed. The data are expressed as a fold changes of the production in the absence of the compounds and the mean $\pm$ SEM for $n = 9 - 11$ (healthy subjects, Figure 8A) or $n = 5$ (asthmatic subjects, Figure 8B) measured in duplicates. *P (**36**) or #P (zileuton **2**) < 0.05 . (P values were calculated using multiple pairwise comparisons of Tukey's procedure.

Boosting of LXA4 levels have been previously reported in an unstimulated isolated platelet/neutrophil cell mixture treated with an LTC4S inhibitor TA05⁶⁵. However, the increase in lipoxins levels on TA05 treatment did not show a consistent and statistically significant dose related response, and only achieved a significant change with the addition of an LTA4H inhibitor. Compound **36** shows a statistically significant dose related increase in lipoxin levels in stimulated whole blood from healthy donors and asthmatics. This confirms the mechanistic observation with

a structurally differentiated LTC₄S inhibitor, and in a more physiological setting of stimulated healthy and asthmatic whole blood. The formation of LXA₄ requires the sequential action of 5-LO and 15-LO, presumably in either order. The observed boosting of LXA₄, while preserving LTB₄ on inhibition of LTC₄S, indicates a more efficient pathway to LXA₄ over LTB₄ in stimulated whole blood. It may suggest a greater intrinsic enzyme efficiency of 15-LO over LTA₄H in processing LTA₄. It may also indicate the complexities of intracellular synthesis vs transcellular metabolic pathways in mixed cell populations. Not all cells possess all the enzymes required to make LTC₄, LTA₄, LXA₄ and LTB₄ from arachidonic acid. For example, platelets do not possess 5-LO but do possess LTC₄S and 15-LO, and must be supplied LTA₄ from other cells⁶⁶. Whereas neutrophils do not contain LTC₄S. There is evidence that endogenously generated arachidonic acid in neutrophils is processed to LTA₄ and LTB₄. However, while exogenously generated arachidonic acid is oxidised to LTA₄ by neutrophils, it is exported from the cell before intracellular LTA₄H can generate LTB₄. This may indicate separate compartmentalised pools of 5-LO and LTA₄H within the neutrophil⁶⁷.

We also hypothesised that an LTC₄S inhibitor would block the formation of eoxins (EXC₄/EXD₄ and EXE₄), products of the 15-LO pathway, as LTC₄S has been shown to be the critical enzyme in forming 14,15-LTC₄ (EXC₄)³⁰ from 14,15-LTA₄ (Figure 1). This was demonstrated with **13**. Compound **13** completely inhibited the formation of EXC₄ from EXA₄ in an LTC₄S enzyme assay in a dose dependent fashion. The IC₅₀ < 25nM was close to the assay limit due to the high LTC₄S enzyme concentration required by the immunoassay protocol, and in agreement with the high LTC₄S potency of this compound. Montelukast is not able to inhibit the production of eoxins in this assay as expected, with an estimated IC₅₀ > 5000nM.

Based on its profile, **36** (AZD9898) has progressed into Phase 1 clinical development and a preliminary account of the Phase 1 program has been published⁶⁸. Overall, single doses of **36** were well tolerated and no safety concerns were raised. Measurement of urinary LTE4 (uLTE4) normalized to creatinine was included as a pharmacodynamic marker to evaluate inhibition of LTC4S. The pharmacodynamic data showed that, at the highest dose tested (45 mg), **36** reduced uLTE4 levels for 14 hours post dose with a maximum reduction at 6 hours post dose. The 45 mg dose was the highest dose possible due to exposure limitation margins. The required magnitude and duration of PD effect at a dose of 45 mg was lower than expected. Furthermore, modelling and simulation of data did not support that the required level of effect on uLTE4 inhibition could be reached and maintained even with twice daily dosing. Therefore, the decision was made to stop the multi-part Ph I study after the single dose phase. In man, the in vivo potency appears lower than predicted from the human PBMC assay and may lie closer to the in vivo efficacy observed in the rat PKPD model. A description and analysis of the development of **36** from candidate drug nomination through to its termination in Phase 1 will appear in a subsequent publication.

Conclusion

While the development of **36** has been terminated early in phase 1 clinical development, its progress has been instructive. A structure-guided and physical property focused approach has yielded a developable candidate LTC4S inhibitor. Drug candidate **36** is 5-fold more potent as an LTC4S inhibitor than the lead compound **7**, but together with a 23-fold reduction in lipophilicity, contributes towards a significant 115-fold improvement in ligand lipophilicity efficiency (LLE), Figure 9 (and Figure S4 which includes compound number labels) This translates to the beneficial in vitro safety, selectivity and rat in vivo profile toxicity of **36** versus **7**.

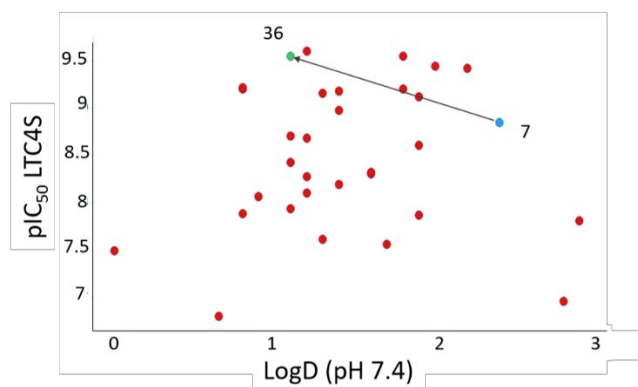


Figure 9. The increase of LTC₄S enzyme pIC₅₀ and reduced lipophilicity of drug candidate (**36**) lead to an improvement in ligand lipophilicity efficiency (LLE) from 6.5 to 8.5 compared to lead compound (**7**) among the compounds tested at AstraZeneca during the LO campaign.

While many projects benefit from iterative crystallography, this project demonstrated the power of a single X-ray structure combined computational chemistry modelling. A rigorous focus on physical properties has solved not just metabolic instability, but also off-target liabilities such as the OATP1B1 transporter interactions which we believed led to the rat sperm duct granulomas observed with lead compound **7**, and GABA binding. These liabilities were resolved in the more hydrophilic final candidate drug. Promiscuity driven by physical properties has become a dogma within medicinal chemistry⁶⁹, but has proven a very robust design criterion within this project. An early in vivo toxicology investigation proved influential in highlighting liabilities with the lead compound **7** which were also mitigated by the focus on reducing bulk lipophilicity and improving LLE, and maintaining structural diversity in the lead optimization process.

Compound **36** showed a direct relationship between exposure and effect in the rat calcium ionophore challenge PKPD model allowing a dose to man prediction to be made based on predicted human pharmacokinetics and the human PBMC potency. The differentiated profile of

1
2
3 compound **36** as an LTC₄S inhibitor, blocking the production of LTC₄/LTD₄ and LTE₄ while
4
5 preserving LTB₄ levels and boosting lipoxin A₄ unlike montelukast and zileuton was
6
7 demonstrated in both healthy and asthmatics whole blood. Equally, as a potent LTC₄S inhibitor it
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9 will be expected to completely block the production of the eoxins, as has been demonstrated with
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11 compound **13**. With an acceptable safety and secondary pharmacology profile and a differentiated
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13 mode of action, compound **36** was judged as suitable for progression into clinical development.
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17 The direct PKPD relationship, and the ease of measurement of urinary LTE₄ as a PD biomarker
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19 allowed monitoring of target engagement in the phase 1 program. It is unfortunate that PKPD
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21 indicated that **36** would not provide level of effect on LTC₄S inhibition required to undertake a
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23 robust phase 2 program to test the mechanistic hypothesis of differentiation from montelukast and
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25 zileuton. However, these data do provide an example of how early evaluation of a PD biomarker
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27 can be utilized for decision making in early clinical development^{70 71}.
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32 We hope that the differentiated profile of an LTC₄S inhibitor shown herein, from other
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34 leukotriene modifier drugs, can indeed at some point fill a therapeutic niche for a subset of
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36 uncontrolled asthma patients, who despite high doses of inhaled corticosteroids, still demonstrate
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38 a very activated leukotriene cascade, as can be identified by high levels of exhaled or urinary
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40 LTE₄.
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EXPERIMENTAL SECTION

General Methods

All solvents and commercial reagents were of laboratory grade and were used as received unless otherwise stated. Reference compounds 1 and 2 were commercial. All temperatures are in degrees Celsius (°C). In general, unless otherwise stated, operations discussed in the below examples were carried out at room or ambient temperature (18 – 25 °C); reaction progress was monitored by HPLC, LCMS, or TLC; oven dried standard laboratory glassware was used and routine manipulations were conducted at ambient temperature under a blanket of nitrogen; evaporations were performed under reduced pressure using a rotary evaporator; and products were dried under reduced pressure at a suitable temperature. Test compounds were purified by silica gel chromatography or by preparative HPLC/SFC and all have a purity greater than 95%. The chirality of all chiral test compounds is greater than 95% ee. **Silica gel chromatography** was carried out using silica gel 60 (0.040-0.063 mm, Merck), or pre-packed Biotage® KP-SIL. Cation/anion exchange columns was Isolute®SCX-2, Isolute®CBA and Isolute®NH₂. **Preparative HPLC** was performed with a Waters FractionLynx system with integrated MS detection or Gilson GX-281 with integrated UV detection using Waters Sunfire C18 OBD 5 µm 19 mm × 150 mm or 30 mm × 150 mm, XBridge BEH C18 OBD 5 µm 19 mm × 150 mm or 30 mm × 150 mm, Xselect CSH C18 OBD 5 µm 19 mm × 150 mm or Kromasil C8 10 µm 20 mm × 250 mm or 50 mm × 250 mm columns and the conditions specified in Table S1. **Preparative chiral HPLC** was performed with a Waters FractionLynx system with integrated MS detection or Gilson GX-281 with integrated UV detection using Chiralpak® IB, Chiralpak® IA or Chiralpak® IB IC 5 µm 20 × 250 mm, Lux® Cellulose-4 5 µm 30 × 250 mm columns and the conditions specified in Table S1. **Preparative SFC** (Supercritical Fluid Chromatography) was performed with a Waters

Prep100 SFC system with integrated MS detection using Chiralpak[®] IC, Chiralpak[®] OJ, Chiralpak[®] AD, Lux[®] Cellulose-2 or Lux[®] Cellulose-4 5 μ m 30 $\times$ 250 mm columns and the conditions specified in Table S1. **LCMS** was run either on Waters Acquity TQD with Waters Acquity UPLC LC/MS/MS System or on Agilent HP 1100 LC/MS system, both equipped with a diode array UV detector from 220 to 350 nm and a mass spectrometer (ESI or APCI) with the following conditions; Waters Acquity UPLC[®] BEH C18 column 1.7 μ m 2.1 mm $\times$ 50 mm, elution gradient with a flow rate of 1 ml/min, 2 – 95% acetonitrile in aqueous 46 mM ammonium carbonate/ammonia buffer at pH 10, or Waters Acquity UPLC[®] HSS C18 column 1.8 μ m 2.1 mm $\times$ 50 mm, elution gradient with a flow rate of 1 ml/min, 2 – 95% acetonitrile in aqueous 10 mM formic acid/ 1 mM ammonium formate buffer at pH 3, or Waters Symmetry[®] C18 column 3.5 μ m 2.1 mm $\times$ 30 mm, elution gradient with a flow rate of 0.7 ml/min, 15–95% acetonitrile in water with 0.1% trifluoroacetic acid over 2.7 minutes, then 95% for 0.3 minutes. **NMR** spectra were recorded on a Bruker Avance, Avance II, or Avance III spectrometer at a proton frequency of 300, 400, 500, or 600 MHz. The central peaks of chloroform δ (H 7.26 ppm), CD₃OD (H 3.30 ppm), or DMSO-d₆ (H 2.49 ppm) were used as internal references. **HPLC purity** determination was made using a Waters Acquity system combined with a Waters Xevo Q-ToF Mass or a Shimadzu 2010EV UPLC system with a mass spectrometer (ESI) equipped with a diode-array UV detector from 220 to 350 nm using Waters Acquity UPLC[®] BEH C18 1.7 μ m 2.1 $\times$ 50 mm, elution gradient with a flow rate of 1 ml/minutes: 5-90% acetonitrile in water with aqueous 46 mM ammonium carbonate/ammonia buffer at pH 10 or Waters Acquity UPLC[®] HSS C18 column 1.8 μ m 2.1 $\times$ 50 mm elution gradient with a flow rate of 1 mL/ minutes: 5-90% acetonitrile in water with 0.1% trifluoroacetic acid. **Optical purity**, indicated as enantiomeric excess (% ee), was assessed with either Thar SuperPure Discovery Series Thar SFC system or Waters Alliance 2696 HPLC system and equipped with a

diode array UV detector from 220 to 350 nm, eluted isocratically with a flow rate of 1 mL/min. SFC conditions are as follows: Lux[®] Cellulose-4 5 μ m 4.6 mm $\times$ 150 mm, elution gradient with 5-40% methanol in CO₂ (40 °C, 150 bar); or Chiralcel[®] OJ 3 μ m 4.6 mm $\times$ 150 mm, 10% ethanol/formic acid 100:0.5 in CO₂ (40 °C, 150 bar); or Chiralpak[®] IC 3 μ m 4.6 mm $\times$ 150 mm, 15% ethanol/formic acid 100:0.5 in CO₂ (40 °C, 150 bar); or Lux[®] Cellulose-2 3 μ m 4.6 mm $\times$ 150 mm, 30% ethanol/formic acid 100:0.5 in CO₂ (40 °C, 150 bar); or Chiralpak[®] AD 3 μ m 4.6 mm $\times$ 150 mm, 15% ethanol/formic acid 100:0.5 in CO₂ (40 °C, 150 bar). HPLC condition: Chiralpak[®] IC 3 μ m 4.6 mm $\times$ 150 mm, heptane/ethanol/formic acid 70:30:0.1; Chiralpak[®] IC 3 μ m 4.6 mm $\times$ 150 mm, heptane/ethanol/formic acid 60:40:0.1; or Chiralpak[®] IC 5 μ m 4.6 mm $\times$ 250 mm, heptane/isopropanol/formic acid 70:30:0.1; or Chiralpak[®] IC 5 μ m 4.6 mm $\times$ 250 mm, heptane/ethanol/trifluoroacetic acid 70:30:0.1. **Optical rotation** was measured with an ADP600 Polarimeters system at the sodium D-line of 589 nm and 20 °C in a sample concentration of 1.0 g/100 mL or 0.5 g/100mL in acetonitrile, methanol or chloroform, and was expressed as $[\alpha]_D^{20}$. **HRMS** (High Resolution Mass Spectrometry) was run on a high resolution (R = 9000 fwhm) LCMS system (Waters Acquity–Xevo Q-ToF) with electrospray ionization (ESI).

Animal Experiments. Rat Ionophore in vivo studies were approved by the local Ethical Committee in Gothenburg (Ethical approval 162-2013). Rat Pharmacokinetic studies were conducted by Pharmaron-bj (AAALAC accredited) in Shanghai, China according to statutes and guidelines enforced at state, province and local levels. Cynomolgus monkey and dog pharmacokinetic studies were performed at Charles River Laboratories and conducted under the UK Home Office Project Licence No. PPL 60/4186 Drug Metabolism and Pharmacokinetics, Protocol Reference Number 3 and ethical approval 1592 (dog PK).

(1*S*,2*S*)-2-{5-[ethyl(4-fluoronaphthalen-1-yl)amino]-3-methoxypyridine-2-**carbonyl}cyclopropane-1-carboxylic acid (16).** NaH (60% in oil) (16 mg, 0.40 mmol) wasadded to **39** (147 mg, 0.360 mmol) in *N,N*-dimethylformamide (2.0 mL) and stirred at 0 °C for 10

minutes. Ethyl bromide (0.054 mL, 0.72 mmol) was added and the mixture was stirred overnight.

The reaction was quenched with water, extracted with ethyl acetate, concentrated and dissolved

in ethanol (3 mL). The mixture was treated with a solution of 3.8 M NaOH (0.830 mL, 3.11

mmol) in water (4 mL) and stirred at 80 °C for 20 minutes. The solution was cooled to room

temperature, acidified with 3.8 M HCl to pH ~3 and extracted with ethyl acetate. The combined

organic layers were concentrated at reduced pressure and the residue was purified by preparative

HPLC. Separation by chiral preparative HPLC gave **16** (32 mg, 22%). ¹H NMR (600 MHz,DMSO-*d*₆) δ 1.21 – 1.25 (m, 3H), 1.26-1.29 (m, 2H), 1.85 (br s, 1H), 3.53 – 3.58 (m, 1H), 3.71(s, 3H), 3.74 – 3.84 (br s, 1H), 3.99 – 4.22 (br s, 1H), 6.60 (s, 1H), 7.31 (s, 1H), 7.50 (dd, *J* =10.3, 8.2 Hz, 1H), 7.58 (dd, *J* = 8.1, 4.9 Hz, 1H), 7.65-7.69 (m, 1H), 7.7 – 7.74 (m, 2H), 8.19 (d,*J* = 8.9 Hz, 1H), 12.41 (br s, 1H). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 12.89, 16.49, 24.76, 26.04,46.91, 55.84, 101.81, 110.92 (d, *J*_{CF} = 20.8 Hz), 121.49 (d, *J*_{CF} = 4.3 Hz), 123.46 (s), 124.71 (d,*J*_{CF} = 17.6 Hz), 126.94, 127.71 (d, *J*_{CF} = 8.8 Hz), 127.92, 128.91, 131.37, 131.85 (d, *J*_{CF} = 5.3Hz), 136.53 (d, *J*_{CF} = 3.8 Hz), 149.31 (s), 157.59 (d, *J*_{CF} = 251.5 Hz), 158.05, 173.82, 194.04.HRMS: *m/z* calcd 409.1563, found 409.1561 [M+H]⁺. [α]_D²⁰ +71 (*c* 1.0, CHCl₃, 589 nm).**(1*S*,2*S*)-2-{5-[(5-chloro-2,4-difluorophenyl)(2-fluoro-2-methylpropyl)amino]-3-****methoxypyrazine-2-carbonyl}cyclopropane-1-carboxylic acid (36).** A solution of NaOH (196mg, 4.92 mmol) in water (3 mL) was added dropwise to a stirred mixture of **44** (231 mg, 0.491

mmol) in methanol (15 mL) at 0 °C for 5 minutes. The resulting mixture was stirred for 15 h. The

reaction mixture was poured into ice-water (100 mL) and the pH was adjusted to 3 - 4 with 2N

HCl. The mixture was extracted with dichloromethane (4×100 mL), dried (Na_2SO_4) and concentrated. Purification by preparative HPLC gave the racemic product (150 mg, 67%) as a yellow solid. LCMS: m/z 458/460 3:1 $[\text{M}+\text{H}]^+$. Separation by chiral preparative HPLC gave **36** (58 mg, 39%) as a white solid. ^1H NMR (600 MHz, DMSO-d_6) δ 1.32 – 1.36 (m, 2H), 1.40 (d, $J = 21.6$ Hz, 6H), 1.89 – 1.96 (m, 1H), 3.52 – 3.58 (m, 1H), 3.83 (s, 3H), 4.26 (d, $J = 21.8$ Hz, 2H), 7.65 (br s, 1H), 7.71 (t, $J = 9.8$ Hz, 1H), 7.98 (t, $J = 7.8$ Hz, 1H), 12.34 (br s, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 16.12, 23.81, 24.32 (br d, $J_{\text{CF}} = 22.4$ Hz), 25.27, 53.41, 57.40 (d, $J_{\text{CF}} = 20.1$ Hz), 96.93 (d, $J_{\text{CF}} = 168.7$ Hz), 106.58 (br t, $J_{\text{CF}} = 27$ Hz), 115.76 (br d, $J_{\text{CF}} = 18.8$ Hz), 121.37, 125.62, 127.81, 130.91, 153.74, 156.31 (dd, $J_{\text{CF}} = 249.3$, 11.9 Hz), 156.90 (dd, $J_{\text{CF}} = 251.9$, 11.2 Hz), 157.76, 173.04, 192.42. HRMS: m/z calcd 458.1094, found 458.1105 $[\text{M}+\text{H}]^+$. $[\alpha]_{\text{D}}^{20} +62$ (c 1.0, CH_3CN , 589).

Ethyl (trans-2-[(5-bromo-3-methoxypyridin-2-yl)-(hydroxy)methyl]cyclopropane-1-

carboxylate (rac-37). 5-Bromo-2-iodo-3-methoxypyridine (2.11 g, 6.72 mmol) and lithium chloride (315 mg, 7.41 mmol) were dissolved in tetrahydrofuran (16 mL), 2M isopropyl magnesium chloride in tetrahydrofuran (3.73 mL, 7.44 mmol) was added dropwise over 30 minutes at -50 $^\circ\text{C}$. The reaction temperature was slowly raised to -20 $^\circ\text{C}$, kept there for 1 h and then cooled to -70 $^\circ\text{C}$ and ethyl trans-formylcyclopropane-1-carboxylate (0.910 mL, 6.89 mmol) was added dropwise. The reaction mixture was allowed to warm to room temperature overnight. Acetic acid (0.853 mL, 14.8 mmol) was added under stirring and the reaction mixture was filtered through a pad of silica gel, washed with ethyl acetate (100 mL) and concentrated under reduced pressure. The residue was purified by silica gel chromatography eluting with ethyl acetate in *n*-heptane (0–80%) to give **rac-37** (840 mg, 38%) as a colourless oil that solidified upon standing. ^1H NMR (500 MHz, DMSO-d_6) δ 0.94 – 0.97 (m, 1H), 1.04 – 1.06 (m, 1H), 1.14

(t, $J = 7.1$ Hz, 3H), 1.56 – 1.60 (m, 1H), 1.79 – 1.84 (m, 1H), 3.87 (s, 3H), 3.95 – 4.05 (m, 2H), 4.62 (t, $J = 6.5$ Hz, 1H), 5.12 (d, $J = 6.9$ Hz, 1H), 7.72 (d, $J = 1.8$ Hz, 1H), 8.25 (d, $J = 1.8$ Hz, 1H).

Ethyl (trans)-2-(5-bromo-3-methoxypyridine-2-carbonyl)cyclopropane-1-carboxylate (rac-38). Manganese dioxide (2.21 g, 25.4 mmol) was added to a stirred solution of **rac-37** (0.840 g, 2.54 mmol) in tetrahydrofuran (18 mL) and heated to 50 °C for 48 h. The reaction was diluted with ethyl acetate (50 mL), filtered through a pad of celite and washed with ethyl acetate (100 mL). The brown solution was concentrated at reduced pressure and purified by silica gel chromatography eluting with ethyl acetate in n-heptane (0 – 40%) to give **38** (0.69 g, 95%) as a yellow oil. ^1H NMR (500 MHz, DMSO- d_6) δ 1.20 (t, $J = 7.1$ Hz, 3H), 1.44 – 1.50 (m, 2H), 2.10 – 2.13 (m, 1H), 3.37 – 3.41 (m, 1H), 3.89 (s, 3H), 4.10 (q, $J = 7.1$ Hz, 2H), 8.01 (d, $J = 1.7$ Hz, 1H), 8.42 (d, $J = 1.7$ Hz, 1H).

Ethyl (trans)-2-{5-[(4-fluoronaphthalen-1-yl)amino]-3-methoxypyridine-2-carbonyl}cyclopropane-1-carboxylate (rac-39). Compound **rac-38** (537 mg, 1.64 mmol), 4-fluoronaphthalen-1-amine (321 mg, 1.96 mmol), Pd(OAc) $_2$ (18.2 mg, 0.082 mmol), racemic BINAP (71.0 mg, 0.110 mmol) and Cs $_2$ CO $_3$ (749 mg, 2.32 mmol) were mixed in toluene (13 mL), purged with nitrogen and stirred in a sealed tube at 95 °C overnight. The reaction mixture was diluted with ethyl acetate (50 mL), filtered through a pad of celite and concentrated under reduced pressure. The residue was purified by silica gel chromatography eluting with gradient ethyl acetate in n-heptane (0-100%) to give **rac-39** (588 mg, 88%) as a brown oil. ^1H NMR (500 MHz, CDCl $_3$) δ 1.24 – 1.27 (m, 5H), 2.26 – 2.32 (m, 1H), 3.68 (s, 3H), 3.78 – 3.84 (m, 1H), 4.14 (q, $J = 7.2$ Hz, 2H), 6.17 (s, 1H), 6.45 (d, $J = 2.1$ Hz, 1H), 7.19 (dd, $J = 8.3, 9.9$ Hz, 1H), 7.41

(dd, $J = 7.9, 4.8$ Hz, 1H), 7.57 – 7.64 (m, 2H), 7.88 (d, $J = 2.2$ Hz, 1H), 7.93 (d, $J = 8.6$ Hz, 1H), 8.19 (d, $J = 8.4$ Hz, 1H). LCMS: m/z 409 $[M+H]^+$.

***N*-(5-chloro-2,4-difluorophenyl)-2-fluoro-2-methylpropanamide (40).** A solution of isobutyl chloroformate (1.10 g, 8.43 mmol) in acetonitrile (10 mL) was added dropwise to a stirred solution of 4-methylmorpholine (0.883 g, 8.68 mmol) and 2-fluoro-2-methylpropanoic acid (0.844 g, 7.91 mmol) in acetonitrile (10 mL) at -10 °C, over a period of 10 minutes under nitrogen. The resulting solution was stirred at -10 °C for 10 minutes. A solution of 5-chloro-2,4-difluoroaniline (1.31 g, 7.93 mmol) in acetonitrile (10 mL) was added dropwise and the resulting solution was stirred at ambient temperature overnight. The reaction mixture was quenched with saturated NaHCO_3 (50 mL), extracted with ethyl acetate (2×100 mL), dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by preparative TLC eluting with petroleum ether/ethyl acetate 10:1 to afford **40** (1.52 g, 75%) as a yellow solid. ^1H NMR (500 MHz, CDCl_3) δ 1.67 (d, $J = 22.3$ Hz, 6H), 6.99 (dd, $J = 10.4, 8.4$ Hz, 1H), 8.25 (br s, 1H), 8.50 (t, $J = 7.9$ Hz, 1H). LCMS: m/z 251/253 3:1 $[M+H]^+$.

5-chloro-2,4-difluoro-*N*-(2-fluoro-2-methylpropyl)aniline (41). A solution of **40** (800 mg, 3.18 mmol) in tetrahydrofuran (70 mL) was cooled to 0 °C and 1M borane tetrahydrofuran complex (15.90 mL, 15.90 mmol) was added dropwise over a period of 5 minutes under air. The resulting solution was stirred at 60 °C for 3h. The reaction mixture was allowed to cool to room temperature and quenched with ice-water (50 mL), acidified with 2N HCl and stirred for 10 minutes. The pH was adjusted to 8 – 9 with saturated NaHCO_3 , stirred for 30 minutes and extracted with dichloromethane (2×100 mL). The organic layer was dried (Na_2SO_4), concentrated under reduced pressure and purified by preparative TLC eluting with petroleum ether/ethyl acetate 25:1 to afford **41** (340 mg, 45%). ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 1.33 (d, $J = 21.5$ Hz,

6H), 3.28 (dd, $J = 21.1, 6.6$ Hz, 2H), 5.65 – 5.72 (m, 1H), 6.96 (dd, $J = 8.9, 7.6$ Hz, 1H), 7.32 (dd, $J = 11.7, 9.2$ Hz, 1H). LCMS: m/z 238/240 3:1 $[M+H]^+$.

Methyl (trans)-2-[methoxy(methyl)carbamoyl]cyclopropane-1-carboxylate (rac-42). Oxalyl dichloride (1.61 g, 12.6 mmol) was added to trans-2-(methoxycarbonyl)cyclopropane-1-carboxylic acid (1.00 g, 6.32 mmol) and *N,N*-dimethylformamide (0.1 mL) in dichloromethane (20 mL) at 0 °C under nitrogen. The resulting solution was stirred at 20 °C for 1 h and concentrated to give the acid chloride (1.00 g, 90%). The product (1.00 g, 5.66 mmol) was dissolved in dichloromethane (10 mL) and added dropwise to a stirred mixture of *N,O*-dimethyl hydroxylamine hydrochloride (0.829 g, 8.49 mmol) and triethylamine (3.16 mL, 22.6 mmol) in dichloromethane (10 mL) at 0 °C during 30 minutes under nitrogen. The resulting mixture was stirred at room temperature for 15 h and washed with water (2 × 50 mL). The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure to give **rac-42** (1.10 g, 97%) as a yellow oil that was used in the next step without further purification. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 1.27–1.34 (m, 2H), 1.94 – 1.98 (m, 1H), 2.55 (br s, 1H), 3.13 (br s, 3H), 3.64 (s, 3H), 3.72 (br s, 3H).

Methyl (trans)-2-(5-bromo-3-methoxypyrazine-2-carbonyl)cyclopropane-1-carboxylate (rac-43). $(\text{CH}_3)_2\text{CHMgCl}\cdot\text{LiCl}$ complex (26.9 mL, 34.9 mmol) was added dropwise to 5-bromo-2-iodo-3-methoxypyrazine (10.0 g, 31.8 mmol) in tetrahydrofuran (300 mL) at -70 °C over a period of 5 minutes under nitrogen. The resulting solution was stirred at -70 °C for 30 minutes. **Rac-42** (5.94 g, 31.8 mmol) in tetrahydrofuran (50 mL) was added at -70 °C. The resulting solution was stirred at -20 °C for 2 h, quenched with saturated NH_4Cl (50 mL) and extracted with ethyl acetate (5 × 100 mL). The combined organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. Purification by silica gel chromatography, eluting with ethyl acetate in

petroleum ether (0–10%) gave **rac-43** (3.60 g, 36%) as a white solid. ¹H NMR (500 MHz, DMSO-d₆) δ 1.48 – 1.53 (m, 2H), 2.14 – 2.18 (m, 1H), 3.46 – 3.50 (m, 1H), 3.6 (s, 3H), 4.0 (s, 3H), 8.6 (s, 1H). LCMS: m/z 315 [M+H]⁺.

Methyl (trans)-2-{5-[(5-chloro-2,4-difluorophenyl)(2-fluoro-2-methylpropyl)amino]-3-methoxypyrazine-2-carbonyl}cyclopropane-1-carboxylate (rac-44). To a stirred solution of **rac-43** (200 mg, 0.634 mmol) and **41** (151 mg, 0.635 mmol) in tetrahydrofuran (10 mL) at -20 °C was added 0.91 M ((CH₃)₃Si)₂NK (0.837 mL, 0.762 mmol, in THF) dropwise over 10 minutes under nitrogen. The resulting mixture was stirred at -20 °C for 30 minutes. The reaction mixture was quenched with ice-water (100 mL), extracted with ethyl acetate (3 × 100 mL), dried (Na₂SO₄), filtered and concentrated under reduced pressure. The residue was purified by preparative TLC eluting with ethyl acetate/petroleum ether 1: 4 to give **rac-44** (237 mg, 79%) as a yellow solid. ¹H NMR (400 MHz, CDCl₃) δ 1.42 (s, 3H), 1.48 (s, 3H), 1.51 – 1.59 (m, 2H), 2.24 – 2.30 (m, 1 H), 3.69 (s, 3H), 3.73 – 3.79 (m, 1H), 4.01 (s, 3H), 4.16 (s, 1H), 4.22 (s, 1H), 7.08 – 7.14 (m, 1H), 7.35 (s, 1 H), 7.50 (t, *J* = 7.6 Hz, 1H). LCMS: m/z 472/474 3:1 [M+H]⁺.

Expression and purification of recombinant LTC4S . A synthetic gene coding for human LTC4S, with an N-terminal 6 × his tag was synthesised (Genscript) and inserted in to the yeast expression vector pPICZB, using the EcoRI and NotI restriction sites. The PmeI linearised expression vector was electroporated into the *Pichia pastoris* X33 strain, following the manufacturers recommendations, and integrants selected on YPD plates containing 100 µg/mL zeocin. Fermentation was carried out as described by Wohri⁷².

120 g of *Pichia pastoris* cells were resuspended in 700 mL of lysis buffer (50 mM tris pH 8.0, 100 mM KCl, 10% glycerol, 1 × complete) and lysed by one passage through a Constant Systems cell disrupter at 40 KPSi. The lysate was centrifuged for 30 minutes at 5000 × g at 4°C. The

supernatant was then pooled and adjusted to 1% Tx100, 0.5% Na-deoxycholate, and the mixture stirred at 4°C. After 4 hrs insoluble material was removed by centrifugation at 15000 × rpm, for 30 mins at 4°C. 40 mL of 50% Ni-Sepharose fast flow resin and 10 mM imidazole was then added and stirred using an overhead mixer for 12 hrs. The resin was harvested by vacuum and packed into an empty XK16 column, and attached to an Äkta Purifier. The resin was washed and eluted in 3 steps: Firstly with a buffer consisting of 25 mM Tris.Cl pH 8.0, 10% glycerol, 0.1% Triton X-100, 1mM TCEP, 0.1 mM GSH, 10 mM imidazole and 0.1 M NaCl until a stable UV280 baseline was obtained. The column was further washed with the same buffer but containing an elevated imidazole (40 mM) and NaCl (500 mM) concentrations. Finally, hLTC4S was eluted from the column by application of 500 mM imidazole in the same buffer. The IMAC fraction of hLTC4S was further purified using affinity purification vi S-Hexylglutathione-Agarose. The protein was loaded onto a 10 mL packed column containing S-Hexylglutathione-Agarose, equilibrated with 25 mM Tris.Cl, pH 7.8, 10% glycerol, 0.1% Triton X-100, 1mM TCEP, 0.5 mM NaCl, 0.1% GSH, after loading the column was washed with the same buffer before elution with 30 mM probenecid. Fractions containing hLTC4S were further purified by size exclusion chromatography, using a Superdex 75 26/60 column, and a running buffer consisting of 20 mM Tris.Cl pH 8, 300 mM NaCl, 0.5 mM TCEP, 0.03% DDM, 10% glycerol. The protein was then concentrated to 5 mg/mL and flash frozen in liquid nitrogen, and stored at -80 °C.

In vitro LTC4 synthase enzyme assay. To compounds (0.1 µl) in dimethyl sulfoxide on a 384 well plate 10 µl of recombinant purified LTC4S was added. After pre-incubation for 15 minutes, the reaction was initiated by addition of 10 µl of substrate solution containing LTA4 methyl ester and glutathione. Final assay conditions were 0.3 nM of LTC4S, 3 µM of LTA4 methyl ester, 0.5 mM glutathione and various concentrations of compound in buffer (0.03% DDM, 0.05% BSA,

100 mM NaCl and 50 mM of Tris-Cl, pH 7.4). After 30 minutes the reaction were stopped by addition of 40 μ l of deionized water:acetonitrile (1:3). The IC₅₀ was determined from a 10-point concentration response curve. Sciex or Waters LC-MS/MS was used for quantitation.

Concentration response to assess species cross-over was setup identical to using 0.3 nM recombinant mouse, rat or guinea pig LTC₄S. Sciex LC-MS/MS system: Sample was injected with CTC analytical injector, Shimadzu LC pumps LC20 and analysed on the Sciex API 5000 LC-MSMS system. Samples were chromatographed on a Thermo HyPurity C18 (3 $\times$ 30 mm, 3 micron particle size) column at a constant flow rate of 1 mL/min. The mobile phases consists of A (water with 0.2% formic acid) and B (acetonitrile with 0.2% formic acid). The LC gradient profile: 10% B during 0 to 0.1 minutes, a linear increase to 100% B during 0.1 to 0.5 minutes, hold at 100% B during 0.5 to 0.9 minutes then back to 10% B from 0.9 to 1 minute. The run time was 1 minutes with retention times of approximately 0.64 and 0.68 minutes for LTC₄ methyl ester and LTA₄ methyl ester, respectively. Detection was performed on a API 5000TM LC/MS/MS system with a triple quadrupole mass spectrometer, a Turbo VTM ion source, in multiple reaction monitoring (MRM) mode at positive polarity. The MRM pairs were m/z 640.4 to m/z 308.1 for LTC₄ methyl ester and m/z 333.3 to m/z 189.0 for methyl ester, respectively.

Waters system: Sample was injected on Waters ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). Liquid chromatography-mass spectrometry was carried out by Waters Xevo TQS triple quadrupole (ESI) with Waters Acquity UPLC interface, chromatographed on a Waters Acquity UPLC HSS T3 column (1.8 μ M particles 2.1 $\times$ 50 mm). The mobile phases consists of A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid). The LC gradient profile: 0.2% B during 0 to 0.3 minutes, a linear increase to 95% B during 0.3 to 1.3 minutes, hold at 95% B during 1.3 to 1.8 minutes then back to 0.2% B from 1.8 to 1.81 minutes. The flow rate is 1 mL/min and retention times of approximately 1.13 and 1.30

minutes for LTC₄ methyl ester and LTA₄ methyl ester, respectively. Detection was performed by the multiple reactions monitoring (MRM) of the transitions. The MRM pairs were m/z 640.4 to m/z 189.1 for LTC₄ methyl ester and m/z 333.2 to m/z 189.1 for methyl ester, respectively.

Statistical analyses of repeat measurements across compounds was undertaken in assay validation, to provide confidence in the assay precision, and to guide decision making in medicinal chemistry. Based on an average of 1 replicate measurement per compound, the 95% confidence interval of pIC₅₀ values was +/- 0.26 log units. This meant that one could be 95% confidence that two compounds show a truly different pIC₅₀ if their potencies differed by 0.37 logunits (2.33 fold on an IC₅₀ scale).

EXC4 assay. The assay used 15 nM final LTC₄ concentration in 75 mM tris buffer pH 8.5, 3 mM GSH / 0.5 mM MgCl₂. The enzyme was preincubated with up to four compounds per 96 well plate to be used for immunoassay detection, at 8 different concentrations in duplicate for 10 minutes. The enzyme reaction was initiated by the addition of EXA4 in diglyme at a final concentration of 8 µM. The total assay volume was 49 µL. After 1 minute the reaction was quenched by adding 49 µL of stop solution (water/methanol/acetic acid 30:70:0.1). The EXC4 formation was detected by EIA (Cayman cat no 10006748) according to the kit instructions. The IC₅₀ was determined from the 8 point dose response curve.

In Vitro Human Peripheral Blood Mononuclear Cell Assay (PBMCs). Blood from human healthy volunteers was collected in EDTA anticoagulant after signed informed consent (Study approved by the local ethics committee in Gothenburg Dnr: 033-10). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation in Ficoll in Greiner Leucosep tubes. The PBMC layer was aspirated and washed in Dulbecco's Phosphate Buffered Saline and finally resuspended in Hank's Buffered Salt Solution.

The PBMC cell suspension, at a cell titer of 75,000 cells/well in a 384 well plate, was pre-incubated at 37 °C with test compound for 45 minutes followed by zymosan activation (200 µg/mL) for 20 minutes at 37 °C. Acetonitrile was added to the assay wells, to stop the reaction, lyse the cells and precipitate proteins, where after LTC4 was measured by LC-MS/MS.

Sciex LC-MS/MS system: Sample was injected with CTC analytical injector, Shimadzu LC pumps LC20 and analysed on the Sciex API 5000 LC-MSMS system with the following settings. Buffer A was 0.2% formic acid in water and Buffer B was 0.2% formic acid in acetonitrile. The LC gradient profile is as follows: 4% B during 0 to 0.1 minutes, a linear increase to 100% B during 0.1 to 0.7 minutes, hold at 100% B during 0.7 to 0.9 minutes then back to 4% B from 0.9 to 0.95 minutes. The flowrate was 1.5 mL/min and a HyPURITY C18, 30×3 column was used for separation of analytes. Detection was performed on a API 5000™ LC/MS/MS system with a triple quadrupole mass spectrometer, a Turbo V™ ion source, in multiple reaction monitoring (MRM) mode at negative polarity. MRM pairs were 624.4 to 271.9 for LTC4.

Data analysis. Genedata Screener was used for curve fitting and calculation of IC₅₀ for LTC4S inhibition. Compound effect was calculated with the formula below;

$$\text{Compound \% effect} = 100 \times ((X - \text{min}) / (\text{max} - \text{min}))$$

where X represents the effect in the presence of test compound, min is dimethyl sulfoxide and max is the maximum inhibition of LTC4S possible (by using compound **7** at a concentration of 2.5 µM). The IC₅₀ values from the PBMC assay were converted to unbound (free) values by multiplying the IC₅₀ with fraction unbound (Fu') in the assay media containing 0.1% BSA, which was calculated according to a principle previously described⁷³, according to the equation:

$$F_u' = \frac{DF \cdot F_u}{1 + (DF - 1) \cdot F_u} \text{ , where } F_u \text{ is fraction unbound in the undiluted condition and } DF \text{ is the}$$

dilution factor of 40. Compound binding to 40 mg/mL (4%) BSA was determined by a standard equilibrium dialysis at a compound concentration of 5 μ M.

Human whole blood assay. Written informed consent was obtained from an exploratory study in asthma patients recruited by Krefting Research Centre, Gothenburg University or healthy volunteers from AstraZeneca. The asthma patient demographic data is shown in Table 6.

Table 6. Asthma patient demographic data for whole human blood study. The patients had mild to severe asthma according to ERS/ATS guidelines⁷⁴ The date of diagnosis for one patient was missing, the FEV1 data were two patients was also not available.

Subjects	1	2	3	4	5
Male/Female	M	M	F	F	F
Age (year)	26	32	66	67	55
Duration of Asthma (year)	6	24	46	16	?
FEV1 (l)	4.29	5.09	2.31	no data	no data
Blood eosinophils (cells/ μ l)	300	100	600	400	200

The study protocols were approved by the local ethics committee in Gothenburg with reference numbers Dnr: 228-14 or Dnr: 033-10 respectively. Human whole blood (HWB) was collected in 10-mL heparinized vacutainer tubes (BD Biosciences) from above study subjects. The HWB was pretreated with or without **36**, montelukast **1** or zileuton **2** at different concentrations for 30 minutes at 37 °C with agitation, followed by incubation for 60 minutes with 10 μ M of calcium ionophore A23187 (Sigma-Aldrich). The reaction was stopped by adding 12.5% of 100 mM EGTA, pH 7.5. The HWB was then centrifuged and plasma was collected for the metabolite

analysis using LC/MS. Samples (50 μ l) were precipitated with acetonitrile containing LTB4-d₄ as an internal standard (180 μ l, 2.6 nM), after vortexing, the samples were centrifuged for 20 minutes at 4000 g at 4 °C and the supernatants (150 μ L) were transferred to a 96-deep well plate and diluted with 75 μ L water. A 20 μ l sample aliquot was analyzed with LC-MS/MS using the following settings. Separation was performed on a 50 $\times$ 2.1 mm Poroshell 120 Bonus-RP column with 2.7 μ m fused core particles (Agilent Technologies Inc., Wilmington, DE, USA) using a gradient elution flow rate at 0.5 mL/min. The mobile phase consisted of (A) 0.2% formic acid in water, and (B) 0.2% formic acid in acetonitrile. The gradient program employed was as follows (given as percentages of mobile phase B): 0 to 1 minutes, 5%, 5 to 6 minutes, 40%, 6.2 to 7.7 minutes, 95%, 8 to 9 minutes, 5%. The gradient was linear between each stated time point. Sample storage and injection was performed with a CTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland). Detection was performed using an AB SCIEX API5500 triple quadrupole (AB SCIEX, Foster City, CA, USA) in multiple reaction monitoring (MRM) negative electrospray ionization mode using the following settings: -4500 ion spray voltage (IS), 25 psi curtain gas (CUR), 60 psi ion source gas 1 (GS1), 90 psi ion source gas 2 (GS2), 8 psi collision gas (CAD), -10 V entrance potential (EP) and 600°C (TEM). The optimized mass transitions and parameters are listed in Table 7. Instrument control, data acquisition and data evaluation were performed using SCIEX Analyst version 1.6.

Table 7. Multiple reaction monitoring (MRM) negative electrospray ionization conditions for all individual compounds and deuterated internal standard

Comp	m/z Q1	m/z Q3	RT (min)	DP (V)	CE (V)	CXP (V)
LTC ₄	624.1	271.8	5.99	-120	-32	-11
LTD ₄	495.1	176.9	5.11	-80	-26	-9
LTE ₄	438.1	333.0	5.80	-70	-24	-15
LipoxinA ₄	351.0	114.8	5.83	-95	-18	-7
LTB ₄	335.0	194.8	6.82	-135	-22	-9
LTB ₄ -d4	339.0	196.8	6.82	-135	-22	-9

Structure determination of LTC₄S. Typically, 150 g cells (20% suspension) were lysed in 25 mM Tris.Cl pH 7.5, 50 mM NaCl, 1 × complete, at 40 kpsi using a Constant Systems cell disrupter. Cell debris was then removed by centrifugation at 10000 × g for 30 mins at 4 °C, followed by collection of the membrane fraction from the supernatant by centrifugation at 42000 rpm in a Beckman Ti45 rotor. Membranes were washed three times by resuspension and centrifugation in ice cold buffer containing 20 mM Tris pH 7.5, 800 mM NaCl, 1 × complete. Optimal conditions for solubilisation of membrane proteins was determined by use of fSEC⁷⁵. Membranes were solubilised in 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 0.5 mM TECP, 1% DDM, for 1 hr with stirring at 4 °C. Insoluble material was then removed by high speed centrifugation and the supernatant applied to a HisTrap Ni Sepharose column using an Äkta Explorer. The column was washed with a buffer consisting of, 20 mM Tris-HCl, pH 8.0, 0.3 M NaCl, 0.5 mM TECP, 0.08% DDM, 15 mM imidazole until the UV280 signal was stable. Bound protein was subsequently eluted by the application of 300 mM imidazole in the same buffer. Fractions containing LTC₄S were pooled and concentrated to 15 mL before separation by size exclusion chromatography on Superdex 200 26/60 column with a mobile phase consisting of 20 mM Tris-

HCl, pH 8.0, 0.3 M NaCl, 0.5 mM TECP, 0.03% DDM. Soluble, pure LTC₄S was concentrated to 10 mg/mL and incubated with 1 mM reduced Glutathione and 1 mM 13 for 2 h before crystallisation. The complex was crystallised using vapour diffusion (1:1 ratio) in 0.2 M CaCl₂, 32% PEG400 and 0.1 M Hepes pH 6.8. The crystal was cryo-cooled and data was collected at the European Synchrotron Radiation Facility, beamLine ID23-1, to 2.35 Å resolution. Data were integrated and processed in space group P2₁2₁2₁ using autoPROC⁷⁶. The structure was solved by molecular replacement using a LTC₄S trimer constructed from the pdb entry 3PCV⁷⁷ as search model. Four trimers of LTC₄S were identified in the asymmetric unit. Structural refinement was carried out in autoBUSTER⁷⁸ and Refmac⁷⁹ and manual rebuilding was done using Coot⁸⁰. Details from the data processing and structure refinement are presented in table S3.

Rat calcium ionophore challenge model. Male Sprague-Dawley rats weighing 230-250 g (Charles River Laboratories, Germany) were orally dosed with vehicle or compound at various times before challenge with vehicle or calcium ionophore A23187. At the time of challenge, the rats were given a lethal dose of pentobarbital intraperitoneally. After the last breath had been taken (after approximately 3 minutes) calcium ionophore A23187 (10 mL vehicle ethanol and PBS) was injected into the lung, and bronchoalveolar lavage fluid was collected by slow aspiration into the syringe. The BALF was immediately centrifuged at 300xg for 10 minutes at 4°C. Supernatants were collected immediately after centrifugation and frozen at -70°C until analysis for cysteinyl leukotrienes (LTC₄/D₄/E₄) and LTB₄. Immediately after injection of the calcium ionophore into the lungs, blood for determination of compound plasma concentration was collected from vena jugularis. The blood samples were centrifuged at 1400 × g for 10 minutes at room temperature, and 45 µL of plasma was transferred to a 96-well plate prepared with 5 µL citric acid (2.14 M) and stored at -70°C until analysis by LC-MS/MS, following protein precipitation.

The % inhibition of CysLT is calculated using the formula:

$$100 - \left(\frac{\text{CysLT}_{\text{treated}} - \text{geomean CysLT}_{\text{vehicle}}}{\text{geomean CysLT}_{\text{A23187}} - \text{geomean CysLT}_{\text{vehicle}}} \right) * 100$$

The relationship between plasma concentration and % inhibition of CysLT was modelled using Phoenix WinNonlin Version 6.4⁸¹, with a sigmoid E_{max} model, according to:

$$E = \frac{E_{\text{max}} \times Cp^{\gamma}}{EC_{50}^{\gamma} + Cp^{\gamma}}$$

, where Cp is the plasma concentration, E_{max} (fixed to 100%) represents the maximum % inhibition, EC_{50} the concentration resulting in half the maximum response and γ describes the sigmoid nature of the concentration-response curve. Unbound EC_{50} is calculated as $EC_{50} \times fu$, where fu is fraction of drug unbound in rat plasma. These studies were approved by the local Ethical Committee in Gothenburg (Ethical Approval 162-2013)

DMPK Assays. DMPK and Safety assays were performed as routine screening assays and have been described in detail elsewhere as indicated.

HPLC logD. Lipophilicity of a compound was determined using reversed-phase liquid chromatography at pH7.4. The logD value was calculated from the correlation between capacity factor and logD as determined for a set of standard compounds in the actual assay set up⁸² (experimental details in eg ⁸³)

ClogP was calculated using the Biobyte software.^{84, 85}

Solubility. The thermodynamic solubility of shortlisted compounds was measured in in PBS pH 7.4. The supernatant samples and the standards of known concentration are evaluated by

LC/MS/MS. Phosphate buffer pH 7.4 is added to a vial plate with the volume of 1 mL per mg solid compound and a stir stick is added to each vial and the vial is sealed with a 96-Well Plate Cover. The Solubility Sample plate is transferred to a plate shaker and shaken at 25°C at 1,100 rpm for 24 h. At the end of incubation, the cover is opened and a record of the phenomena is taken for each vial. The plate is centrifuged at 4,000 rpm, 25°C for 30 minutes and an aliquot of 750 µL is transferred from the supernatant and subsequently dispensed into another 96-vial glass insert plate and centrifuged again (4,000 rpm, 25°C, 30 minutes). An aliquot of 10 µL is transferred from the Solubility Sample plate after double centrifugation to a new 96-well plate by addition of 990 µL of a mixture of H₂O and acetonitrile containing internal standard (1:1) (100 folds). 10 µL of the diluted solution is pipetted into a new 96-well plate by addition of 990 µL of a mixture of H₂O and acetonitrile containing internal standard (1:1) (10,000 folds). The samples were analyzed and quantified against the standard of known concentration using LC-MS/MS. The solubility of the test compound is calculated as follows:

$$[\text{Sample}] = \text{Area Ratio sample} \times \text{DF sample} \times [\text{STD}] / \text{Area Ratio STD. DF is the dilution factor.}^{82}$$

CYP inhibition. Cytochrome P450 enzyme inhibition was performed in a semiautomated assay using human recombinant CYPs and coumarin based substrates that are biotransformed to fluorescent metabolites. The inhibitory action for each CYP was determined at five different compound concentrations and the IC₅₀ value was calculated accordingly.⁸⁶

Hepatocyte CL_{int} (human, rat, dog). Metabolic stability was determined by incubating 1 µM compound solution in cryopreserved hepatocytes (1 million cells/mL), at 37°C and pH 7.4. Compound concentration at different time points (typically 2, 15, 30, 45 and 60 min) was quantified by LC-MS/MS and CL_{int} calculated from compound disappearance.⁸⁷ Pooled human, rat (Han

wistar) and dog (Beagle) hepatocyte batches were used as available for screening at the time of assay.

Caco2 P_{app}. The assay has been described in detail elsewhere⁸⁸. An automated assay to study Caco-2 monolayer permeability in the apical to basolateral direction including a pH gradient (pH 6.5 in apical donor compartment and pH 7.4 in basolateral receiver compartment) was used.

For efflux determination two experiments were performed in a similar setup, but without pH gradient (pH 7.4 in both apical and basolateral compartment). Efflux ratios were calculated as the ratio of the apparent permeability coefficient in the apical to basolateral direction and the apparent permeability coefficient in the basolateral to apical direction:

$$ER = P_{appB-A} / P_{appA-B}$$

Reactive Metabolite trapping (GSH). Compounds were incubated in human liver microsomes in the presence of the trapping agent (glutathione) and NADPH at pH 7.4 and 37 °C. Adducts were identified by QTOF MS analysis and Waters Metabolynx software used for processing and data interpretation. For GSH adducts a ranking based on the 'GSH ratio' (=total integrated peak area of glutathione adducts of the test compound divided by the integrated peak area of the major glutathione adduct of the control compound clozapine) was possible.

OATP1B1. OATP1B1 transporter inhibition was performed in OATP1B1 expressed HEK293 cells using Estradiol-17β-glucuronide as probe substrate. Cell culturing and incubations performed as described by Brännström et al⁸⁹

GABA_{A1} binding was performed by Cerep using radioligand displacement of [3H]muscimol and performed in CHO cells, as described by Wang⁹⁰

Plasmaprotein binding (human, rat, dog). Plasma protein binding experiments were performed as reported previously⁹¹ using blood plasma samples from humans, Sprague Dawley rats, Beagle dogs, and cynomolgus monkey.

In vivo PK experiments in rat and dog (clearance CL, half-life $t_{1/2}$, steady state volume of distribution Vss, bioavailability F%). **Rat:** pharmacokinetic properties of selected compounds were studied in male Han Wistar rats. Plasma pharmacokinetics were determined from 0-24h following iv or po administration, calculated as mean from two animals. Doses were usually 0.5 mg/kg for iv and 1 mg/kg for po experiments. **Dog:** pharmacokinetic properties of selected compounds were studied in male Beagle dogs. Plasma pharmacokinetics were determined 0-48h following iv or po administration and parameters calculated as mean from two animals. Doses were 1 mg/kg iv, administered as a 15 minutes infusion and 2 mg/kg oral gavage. **Cynomolgus monkey:** pharmacokinetic properties of selected compounds were studied in cynomolgus monkeys. Plasma pharmacokinetics were determined from 0-48h following iv or po administration, calculated as mean from three animals. Doses were 1 mg/kg for iv and 2 mg/kg for po experiments.

hERG inhibition. hERG inhibition was determined using the IonWorks™ instrument as was described earlier.⁹²

Inhibition of other cardiac ion channels. Inhibition of four human ion channels, hNav1.5, hCav1.2, hKvLQT1/hminK (IKS) and hKv4.3-hKChIP2.2 (Ito), included in a cardiac screening panel, was determined using the IonWorks procedure as described by Davies et al.⁹³

AMES assays. AMES activity determination was performed as described earlier.⁹⁴

ASSOCIATED CONTENT

Supporting information

The Supporting Information is available free of charge on the ACS Publication Website at DOI:

Genral synthetic information, detailed synthetic chemistry procedures for compounds **5-11**, **13-15** and **17-35**, X-ray diffraction and determination of absolute configuration of **36**, Quantum Mechanic calculations for core variations, crystallographic data collection and refinement statistics for **12** modelled and refined in *R,R* and *S,S* configuration and a version of Figure 9 with all compound numbers in this paper (PDF)

Molecular Formula Strings, LTC4S IC₅₀ and PBMC IC₅₀ (CSV)

Authors will release the atomic coordinates and experimental data upon article publication (pdb 6R7D)

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

AA, arachidonic acid; BALF, bronchoalveolar lavage fluid; BINAP, 2,2'-bis(diphenylphosphino)-1,1'-binaphthyl; BSEP, bile salt export pump assay; Caco-2, colorectal adenocarcinoma cell-2; Caco-2 Papp, colorectal adenocarcinoma cell-2 apparent permeability; CHO cells, chinese hamster ovary cells; CL, clearance; Clint, intrinsic clearance; CV, coefficient of variation; CysLT, cysteinyl leukotrienes; CysLTR1, cysteinyl leukotriene receptor 1; CysLTR2, cysteinyl leukotriene receptor 2; DDM, n-dodecyl β -D-maltoside; $E_{\max}$, maximum effect; EXA4, 14,15-leukotriene A4, eoxin A4; EXC4, 14,15-leukotriene C4, eoxin C4; EXD4, 14,15-leukotriene D4, eoxin D4; EXE4, 14,15-leukotriene E4, eoxin E4; FLAP, 5-lipoxygenase

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3 activating protein; GSH, glutathione; GPR99, oxoglutarate receptor 1 also known as cysteinyl
4
5 leukotriene receptor E; Huhep, human hepatocyte; HWB, human whole blood; 5-LO, 5-
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7 lipopxygenase; LTA4, leukotriene A4; LTB4, leukotriene B4; LTA4H, leukotriene A4 hydrolase;
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9 LTB4, leukotriene B4; LTC4S, leukotriene C4 synthase; LTC4, leukotriene C4; LTD4,
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11 leukotriene D4; LTE4, leukotriene E4; LO, lead optimization; LE, ligand efficiency; LLE, ligand
12
13 lipophilic efficiency; LXA4, lipoxin A4; MRM, multiple reaction monitoring; MRP2, multi-drug
14
15 resistance associated protein 2; OATP1B1, organic anion transporter 1B1; PBMC, peripheral
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17 blood mononuclear cell; PKPD, pharmacokinetic-pharmacodynamic; SEM, standard error of the
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19 mean; STD, standard; TCEP, tris(2-carboxyethyl)phosphine; THLE cell, T-antigen immortalized
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21 human liver epithelial cell line; UPLC, ultra-performance liquid chromatography; Vss, volume of
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23 distribution at steady state; Xantphos, 4,5-Bis(diphenylphosphino)-9,9-dimethylxanthene;
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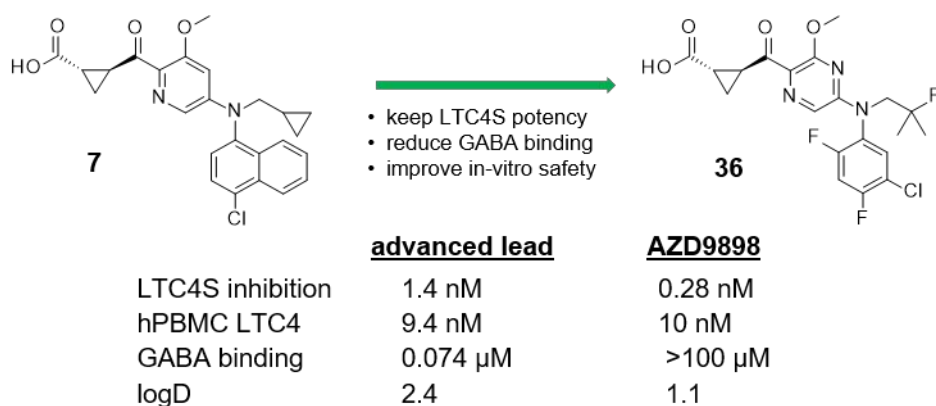
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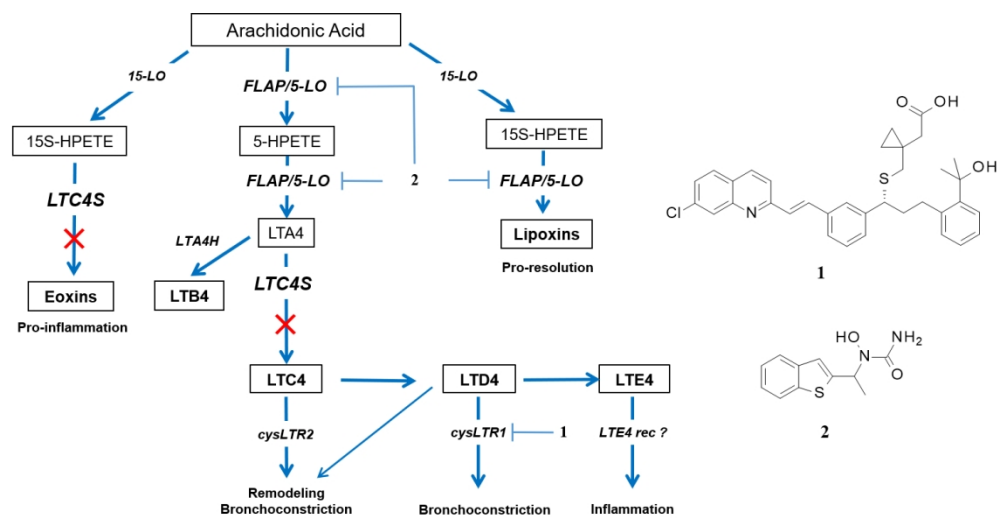


Figure 1. The differentiation in the arachidonic acid cascade of an LTC4S inhibitor (marked with a red x) compared to a cysteinyl leukotriene receptor 1 antagonist, montelukast 1 and a 5-lipoxygenase inhibitor zileuton 2.

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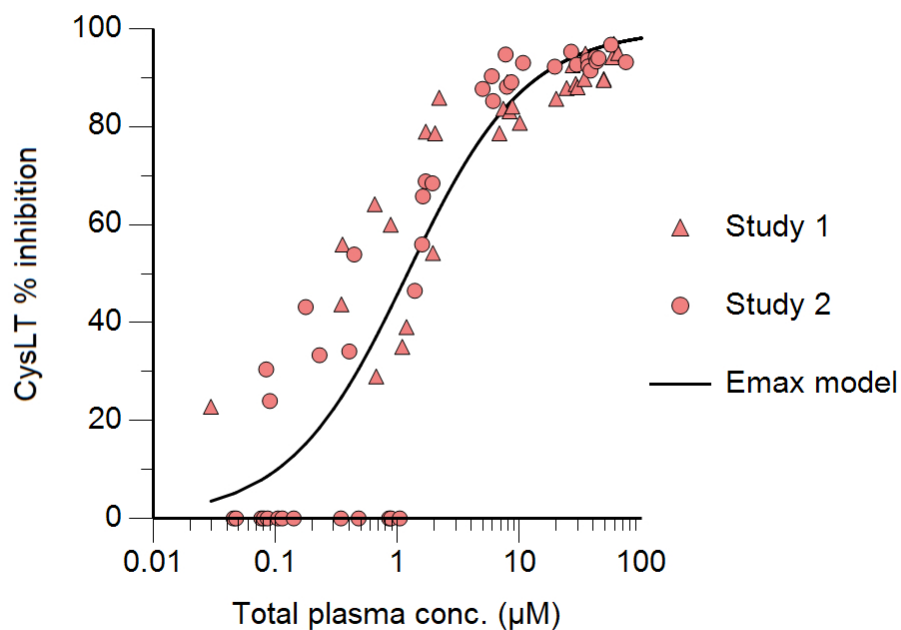


Figure 3 Effect of 7 on inhibition of CysLT production in the rat calcium ionophore challenge model. Compound 7 was administered orally two hours prior to a calcium ionophore challenge with A23187 via terminal bronchoalveolar lavage (BAL), and plasma concentration of 7 was determined at time of PD (CysLT in BAL fluid) measurement. Data from two consecutive experiments (1-90 mg/kg in study 1, triangles; 0.1-90 mg/kg in study 2, circles) were pooled and fitted to a sigmoid Emax model, yielding an EC₅₀ of 1.21 μM (CI 95% 0.89-1.5).

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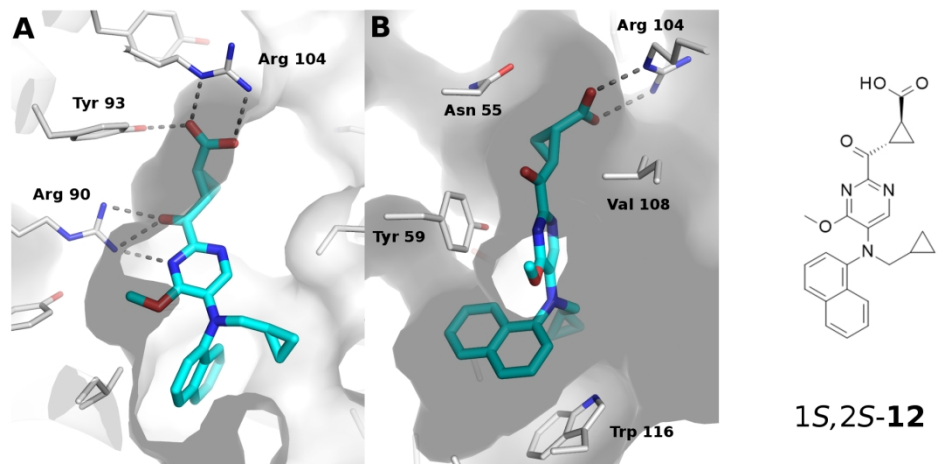


Figure 4. X-ray crystal structure of 1S,2S-12 complexed with human LTC₄S (pdb 6R7D). (A) The acid forms hydrogen bonds to the side chain of Arg104 and the phenol oxygen on Tyr93. The side chain of Arg90 is arranged to coordinate the keto-group and one of the pyrimidine nitrogens. N-cyclopropyl methyl side chain faces the entrance of the binding pocket (B) Tyr59 π -stacked with the pyrimidine ring.

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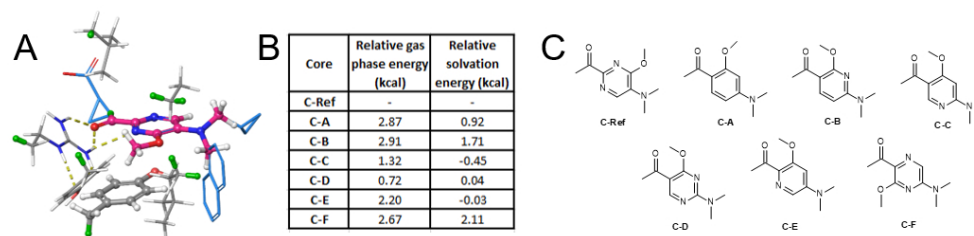


Figure 5. (A) Reduced system for QM calculations with the core ring (magenta) of ligand 12 (blue) with selected amino acids (grey) based on the X-ray of 12. Atoms fixed during the geometry optimization are colored green. (B) Relative energies are given between the reference core ring from 12 (C Ref) and the modelled variants. (C) Core ring models used in the QM calculations

279x66mm (96 x 96 DPI)

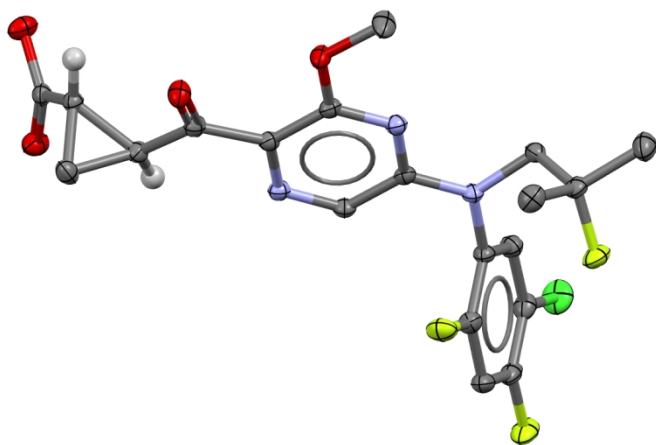


Figure 6. Single crystal X-ray diffraction structure of 36. Thermal ellipsoids drawn at the 50% probability level. Hydrogen atoms and methanol moiety are omitted for clarity.

438x193mm (96 x 96 DPI)

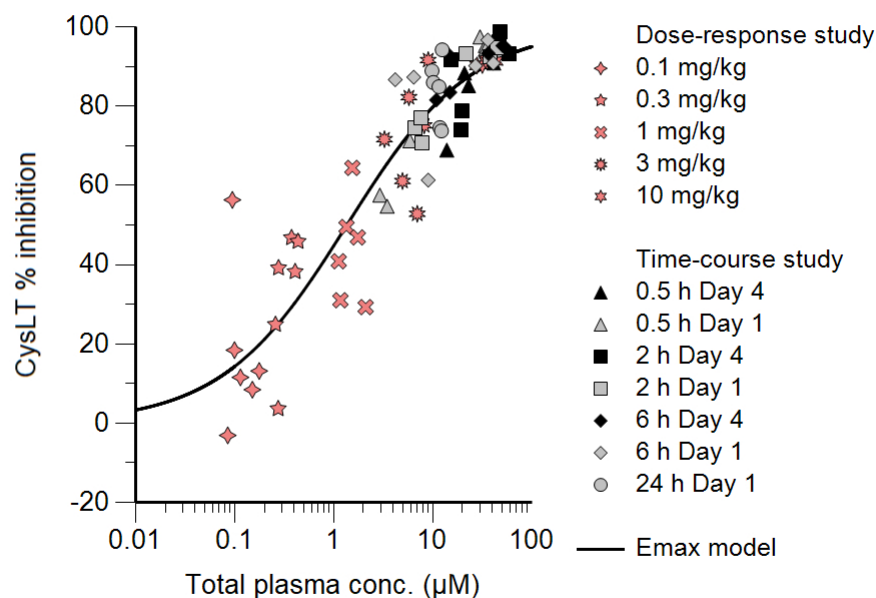


Figure 7. Effect of 36 on inhibition of CysLT production in the rat calcium ionophore challenge model. In the dose-response study 36 was administered orally two hours prior to a calcium ionophore challenge via BAL. In the time-course study, the challenge was done 0.5, 2, 6 or 24 hours post a single dose, and 0.5, 2 or 6 hours after four days of daily oral dosing at 3 or 10 mg/kg. Data across both studies were pooled and fitted to a sigmoid Emax model, yielding an EC₅₀ of 1.36 μM (CI 95% 0.99-1.7).

264x226mm (96 x 96 DPI)

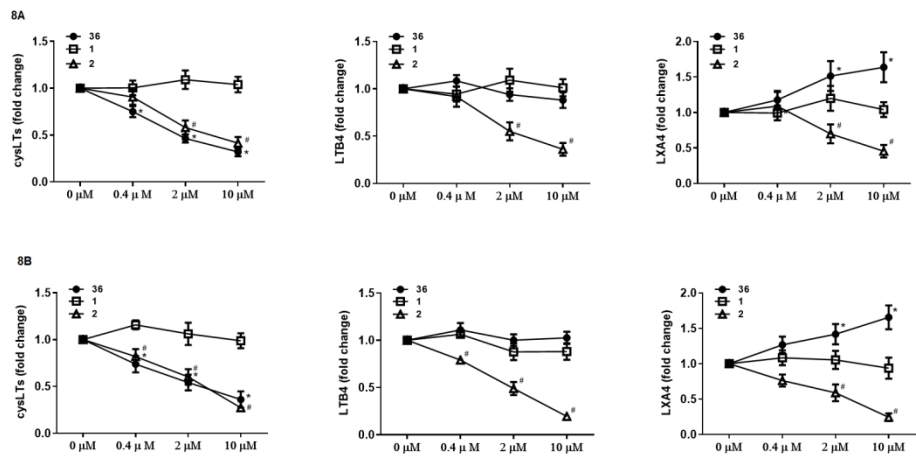


Figure 8. Compound 36 regulates the arachidonic acid (AA) pathway differently from montelukast 1 and zileuton 2 in human whole blood. Metabolite productions stimulated by calcium ionophore in the absence or presence of different concentrations of 36, montelukast 1 or zileuton 2 were analysed. The data are expressed as a fold changes of the production in the absence of the compounds and the mean + SEM for n = 9 – 11 (healthy subjects, Figure 8A) or n = 5 (asthmatic subjects, Figure 8B) measured in duplicates. *P (36) or #P (zileuton 2) < 0.05. (P values were calculated using multiple pairwise comparisons of Tukey’s procedure.

365x175mm (120 x 120 DPI)

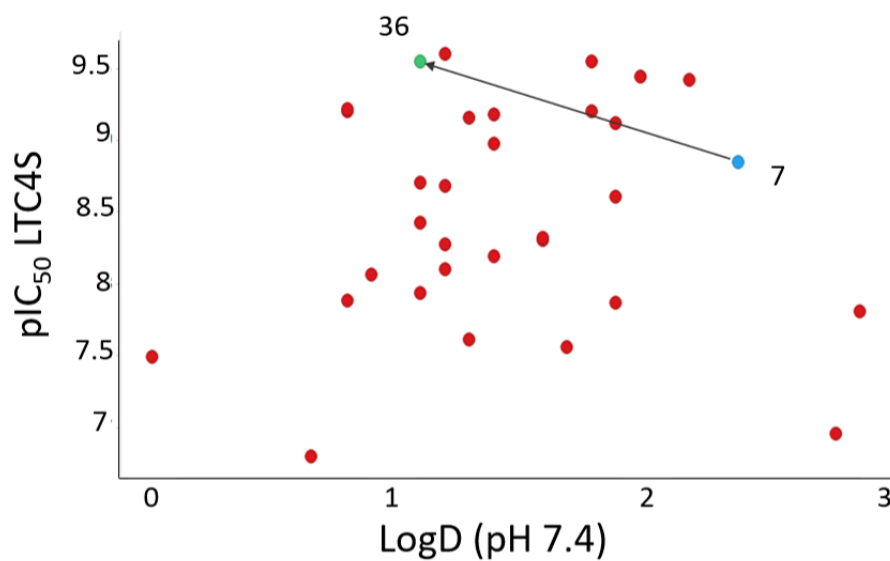
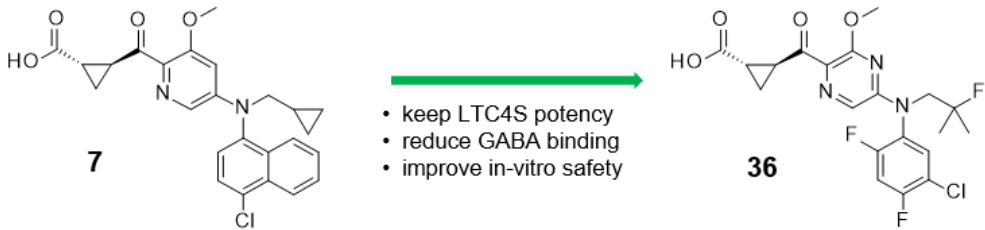


Figure 9. The increase of LTC_{4S} enzyme pIC₅₀ and reduced lipophilicity of drug candidate (36) lead to an improvement in ligand lipophilicity efficiency (LLE) from 6.5 to 8.5 compared to lead compound (7) among the compounds tested at AstraZeneca during the LO campaign.

247x152mm (96 x 96 DPI)



- keep LTC4S potency
- reduce GABA binding
- improve in-vitro safety

	<u>advanced lead</u>	<u>AZD9898</u>
LTC4S inhibition	1.4 nM	0.28 nM
hPBMc LTC4	9.4 nM	10 nM
GABA binding	0.074 μ M	>100 μ M
logD	2.4	1.1

TOC

196x86mm (96 x 96 DPI)