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Research paper

4,5-Diarylisoxazol-3-carboxylic acids: A new class of leukotriene biosynthesis inhibitors potentially targeting 5-lipoxygenase-activating protein (FLAP)

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ABSTRACT

In this article, we report novel leukotriene (LT) biosynthesis inhibitors that may target 5-lipoxygenase-activating protein (FLAP) based on the previously identified isoxazole derivative (**8**). The design and synthesis was directed towards a subset of 4,5-diaryl-isoxazole-3-carboxylic acid derivatives as LT biosynthesis inhibitors. Biological evaluation disclosed a new skeleton of potential anti-inflammatory agents, exemplified by **39** and **40**, which potently inhibit cellular 5-LO product synthesis ($IC_{50} = 0.24$ μM, each) seemingly by targeting FLAP with weak inhibition on 5-LO ($IC_{50} \geq 8$ μM). Docking studies and molecular dynamic simulations with 5-LO and FLAP provide valuable insights into potential binding modes of the inhibitors. Together, these diaryl-isoxazol-3-carboxylic acids may possess potential as leads for development of effective anti-inflammatory drugs through inhibition of LT biosynthesis.

1. Introduction

Leukotrienes (LTs) are potent lipid mediators that play important roles in the pathophysiology of inflammatory, fibrotic and hyperproliferative diseases such as asthma, chronic obstructive pulmonary disease (COPD), inflammatory bowel disease (IBD), arthritis, allergic diseases, autoimmune diseases, cardiovascular disease (CVD) and certain cancer types [1,2]. In the first step of LT biosynthesis, 5-lipoxygenase (5-LO) catalyzes the production of the unstable epoxide LTA_4 from arachidonic acid (AA), which is further metabolized to LTB_4 or cysteinyl LTs (cys-LTs) such as LTC_4, D_4 and E_4 [3,4]. This first step also requires the involvement of 5-LO-activating protein (FLAP), which acts as a regulatory protein by interaction with 5-LO for the transfer of AA to 5-LO for efficient metabolism [5].

Despite intensive efforts to develop 5-LO/FLAP inhibitors as anti-LTs, zileuton (**1**), the only 5-LO inhibitor, reached the market for the treatment of asthma and/or allergic rhinitis, but has experienced limited use due to its poor pharmacokinetics and observed idiosyncratic hepatotoxicity [6]. Additionally, the 5-LO inhibitor setileuton (**2**, MK-0633) [7–9] and several FLAP inhibitors such as AM803 (**3**, GSK2190915), AZD6642 (**4**) and BI665915 (**5**) were reported to be in various stages of preclinical and clinical studies for treatment of asthma and COPD [10–13].

Recent studies have also implicated LTs in CVD such as atherosclerosis, myocardial infarction (MI), and stroke [14,15]. 5-LO and FLAP are considered to be important components of the LT cascade found in atherosclerotic lesions, thus implicating their involvement in atherogenesis [16–18]. The 5-LO inhibitor **2** recently completed phase II clinical trials for atherosclerosis (Clinical trials ID: NCT00421278) as well as for asthma and COPD [7,19], implying the value of LT inhibition for CVD besides respiratory disorders.

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Pharmacological intervention with FLAP has also been reported to decrease atherosclerotic lesion size in animal models [20–23] and the FLAP inhibitor veliflapon (**6**, DG-031/BAY-X1005) has successfully passed a phase II human clinical trial for MI, although participant recruitment in phase III was suspended due to unexpected formulation issues. In addition, there is a growing interest in 5-LO or FLAP inhibitors as broad-spectrum LT biosynthesis inhibitors in cancer therapy, especially in prostate cancer [24,25] and certain types of leukemia [26–28].

Inspired by the therapeutic potential of LT biosynthesis inhibitors, we have recently introduced two promising chemotypes, a benzimidazole derivative 1-(2-chlorobenzyl)-2-(1-(4-isobutylphenyl)ethyl)-1*H*-benzimidazole (**7,** BRP-7) and an isoxazole derivative 2-[4-(4-chlorophenyl)-3-methyl-1,2-oxazol-5-yl]-5-[(2-methyl phenyl)methoxy]phenol (**8**) as anti-LT agents [29,30] (see Fig. 1). From the SARs [29,31] and detailed pharmacological studies [32], **7** was revealed as potent inhibitor of LT biosynthesis targeting FLAP in a cell-based assay (IC_{50} = 0.15–0.31 μM) without affecting isolated 5-LO (IC_{50} > 10 μM), whereas **8** was moderately effective in a cell-based assay (IC_{50} = 4.4 μM) and directly inhibited isolated 5-LO (IC_{50} = 1.9 μM), which might be the primary cause for the decrease of cellular LT formation [29]. However, whether **8** also interferes with FLAP was not explored in those studies. Therefore, the present work describes the structure-guided design, synthesis, structure–activity relationship (SAR), and biological evaluation of novel 4,5-diarylisoxazol-3-carboxylic acids as potent inhibitors of LT biosynthesis, potentially targeting FLAP.

2. Results and discussion

2.1. Docking studies and molecular dynamic (MD) simulations with 5-LO

Based on the favorable profile of **8**, we focused on the preparation of further derivatives in order to understand SARs for interference with 5-LO product synthesis. First, we addressed the necessity of the *o*-hydroxyl group on C5-phenyl for the bioactivity of **8**. As we previously showed the importance of hydrophobic contacts over hydrophilic interactions as a general determinant for many known 5-LO inhibitors [33], our initial hypothesis was the removal of the *o*-hydroxyl group to improve the potency. Therefore, we synthesized two derivatives of **8** by either completely removing the polar *o*-hydroxyl moiety (**15**) or by replacing it by a less polar methoxy group (**16**) (Scheme 1). Interestingly, removal of the *o*-hydroxyl group from **8** or *O*-methylation abolished the inhibition of cellular LT biosynthesis (**15**–**16**, Table 1).

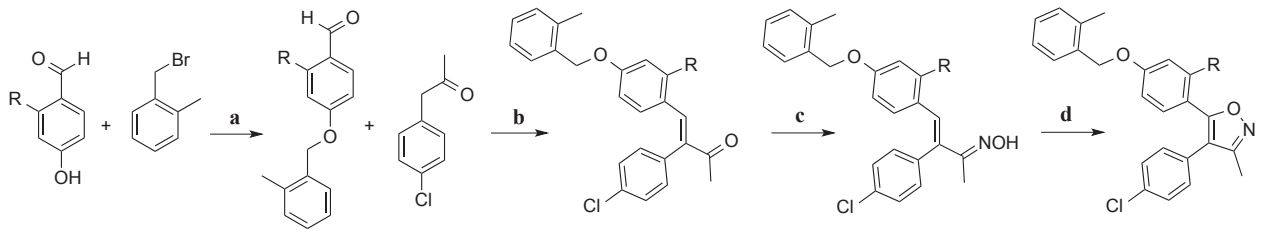
For rationalizing the inhibitory activity of **8** on 5-LO by means of molecular docking, we used the recently reported apo form of the 5-LO crystal structure (PDB code 3O8Y). The unavailability of a holo 5-LO conformation represented the first major difficulty to elucidate the ligand-active site interactions. Therefore, we referred to the conserved or unconserved active site interaction models [33,34]. Grounding on those results, we combined docking studies with MD simulations (see Supporting information for detail) to investigate the binding mode of inhibitors taking into account ligand-induced conformational changes of 5-LO. As a result of the docking studies, **8** interacts with the binding site engaging residues Tyr181, Trp599, Phe177, His372, Phe421, Asn425 and His600 (Fig. S1). The 5-LO/**8** complex obtained by docking was submitted to a 10 ns MD study (Fig. 2A). As a result of MD simulations, the *o*-hydroxyl group is engaged in a stable hydrogen bonding interaction with the side-chain of Asn425 (occupancy, 94%, Fig. 2A) orienting the phenyl moiety to engage π-π contacts with Tyr181 (occupancy, 38%), His372 (occupancy, 39%) and Phe421 (occupancy, <30%), which is in agreement with previously reported data on the catalytic site of 5-LO [35].

These findings led us to turn our attention into a structure-based design of new derivatives of **8** involving the isoxazole-3-carboxylic acid core (**32**, Fig. 2B), where the carboxyl arm on isoxazole might influence the pattern of more strong interactions with neighboring polar amino acids or with amino acid backbones. Indeed, from the analysis of molecular docking and MD results, both the isoxazol-3-carboxylic acid analog **32** and **8** disclosed a similar binding mode at the 5-LO active site (Fig. 2A–B). Compound **32**, as already seen for the compound **8**, engaged a strong hydrogen bond reinforced by charge assistance with Asn425 (occupancy, 99%), as well as maintaining the key hydrophobic contacts with Tyr181 (occupancy, <30%), His372 (occupancy, 34%) and Phe421 (occupancy, 32%). These results suggested the synthesis of a focused library of compounds with decorations on an isoxazole-3-carboxylic acid core that was designed taking into account both synthetic accessibility, and the compatibility of new skeleton with the binding requirements of the active pocket to obtain preliminary SAR (Scheme 2). The biological evaluation of this small set of compounds might be helpful for the comprehension of the key features of new isoxazole-3-carboxylic acids as LT biosynthesis inhibitors.

2.2. Chemistry

Compounds **15**–**16** were prepared following the reaction sequence shown in Scheme 1 using general methods previously reported [36]. Hence, the α,β-unsaturated ketone intermediates (**11**–**12**), which were prepared by condensation of 4-chlorophenylacetone with benzaldehydes (**9**–**10**) in the presence of piperidine, were converted to α,β-unsaturated oximes (**13**–**14**). Subsequent cyclization of the oximes (**13**–**14**) generated the 3-methylisoxazole derivatives (**15**–**16,** Scheme 1).

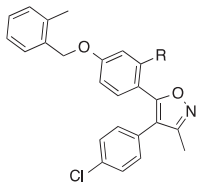
For the synthesis of compounds **32**–**40**, we utilized the



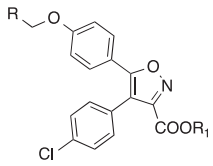
9 R = H
10 R = OCH_3
11 R = H
12 R = OCH_3
13 R = H
14 R = OCH_3
15 R = H
16 R = OCH_3

Scheme 1. Synthesis of 4,5-diaryl-3-methylisoxazoles. Reagents and conditions: (a) K_2CO_3, MeCN, reflux; (b) piperidine, 90 °C; (c) $NH_2OH.HCl$, NaOAc, EtOH, reflux; (d) KI, I_2, $NaHCO_3$, THF, H_2O, reflux.

Table 1
Effects of test compounds on 5-LO product synthesis in a FLAP-dependent cell-based (intact neutrophils) and in a FLAP-independent cell-free (purified 5-LO) assay.



8, 15-16



23-25, 32-40

Cmpd	R	R_1	5-LO product formation		
			Cell-based assay[a] (% remaining activity at 10 μM)	IC_{50} [μM][b]	
				Cell-based	Cell-free
8	OH		3.0 ± 0.7	4.4 ± 1.1	1.9 ± 0.5
15	H		101.5 ± 5.4	n.d.	n.d.
16	OCH_3		81.3 ± 8.2	n.d.	n.d.
23	2-MePh	Et	71.9 ± 10.3	n.d.	n.d.
24	3-FPh	Et	55.3 ± 12.1	n.d.	n.d.
25	4-MePh	Et	61.4 ± 12.4	n.d.	n.d.
32	2-MePh	H	13.1 ± 3.9	1.7 ± 0.5	0.48 ± 0.03
33	3-FPh	H	19.6 ± 9.5	1.3 ± 0.6	0.38 ± 0.04
34	4-MePh	H	15.7 ± 4.5	1.4 ± 0.1	5.9 ± 0.4
35	3-CNPh	H	6.9 ± 3.9	4.1 ± 0.3	16.3 ± 5.6
36	2-ClPh	H	5.1 ± 1.5	1.2 ± 0.2	6.1 ± 0.9
37	2-CF_3Ph	H	5.5 ± 2.2	0.77 ± 0.2	6.0 ± 0.7
38	pyridin-2-yl	H	8.4 ± 0.7	4.1 ± 0.1	7.4 ± 2.5
39	quinolin-2-yl	H	2.7 ± 1.4	0.24 ± 0.07	8.0 ± 3.0
40	benzothiazol-2-yl	H	2.0 ± 0.8	0.24 ± 0.10	>10
1	zileuton		6.7 ± 2.1	0.58 ± 0.11	0.91 ± 0.25

Compounds were tested for inhibition of 5-LO product formation in human neutrophils stimulated with 2.5 μM A23187 (cell-based assay) and against purified human recombinant 5-LO (cell-free assay).
[a] Data are given as remaining 5-LO activity as percentage of uninhibited control (100%, containing 0.3% DMSO as vehicle) at 10 μM inhibitor concentration (means ± SE, n = 3–4). The 100% value of the control corresponds to an average of 30 ± 6 ng 5-LO products per 10^6 neutrophils.
[b] The IC_{50} values are given as mean ± SE of n = 3–4 determinations in duplicates. n.d. not determined.

synthetic procedure outlined in Scheme 2, following published standard procedures [37]. In brief, the commercially available *p*-hydroxyacetophenone was protected with benzyl group (**17**) prior to the subsequent reaction to generate a keto-enol ester (**18**) by reaction with diethyl oxalate. Formation of the isoxazole ring **19** was done by reaction of **18** with hydroxylamine in ethanol. Bromination at the 4-position of the isoxazole (**20**) was achieved with *N*-bromosuccinimide (NBS) in the presence of a catalytic amount of ceric ammonium nitrate (CAN). Compound **20** underwent a palladium-catalyzed Suzuki cross-coupling reaction with *p*-chlorophenylboronic acid to afford **21**. Hydrogenation of **21** to remove benzyl group furnished the intermediate **22**, which was subsequently used to produce desired final compounds **32**–**40** through first the alkylation of the phenolic hydroxyl and then the hydrolysis of the ester group. All compounds were purified by recrystallization or by automated flash chromatography and checked for purity by TLC and UPLC before being tested in biological assays (purity was ≥95% based on the peak area percentage of UPLC analysis). The structures of the compounds were confirmed by high-resolution mass spectrometry (HRMS), elemental analysis, ^1H and ^{13}C NMR spectral data.

2.3. Biological evaluation and SAR studies

LT biosynthesis is initiated by cytosolic phospholipase A_2 ($cPLA_2$)-mediated release of AA, which is subsequently converted to LTA_4 by activated 5-LO with the aid of FLAP that may facilitate access of 5-LO to AA [2]. While FLAP is essential for cellular 5-LO product formation from endogenous AA, it is dispensable for 5-LO activity in cell-free systems [38]. Therefore, we investigated the test compounds in a 'FLAP-dependent' cell-based assay for suppression of 5-LO product formation using Ca^{2+}-ionophore A23817 activated neutrophils, as well as in a 'FLAP-independent' cell-free 5-LO activity assay using human recombinant 5-LO and 20 μM AA as exogenous substrate. Analyzed 5-LO products include 5-H(P)ETE and all trans-isomers of LTB_4, as well as LTB_4 in intact cells. The direct 5-LO inhibitor zileuton (**1**) was used as reference drug.

Starting from **8** as hit compound, we aimed to explore (*i*) the influence of differently positioned substituents on the benzyloxy arm, (*ii*) the replacement of benzyloxy by heteroaryl moieties, and (*iii*) the role of carboxylic acid or ester groups at position 3 of the isoxazole for inhibition of 5-LO product synthesis (Fig. 3).

First, we prepared the 3-carboxyl analogue of the inactive **15** yielding **32**, which was highly active and even outperformed hit compound **8**, as the potencies of **32** in the cell-free (IC_{50} = 0.48 μM) and cell-based assay (IC_{50} = 1.7 μM) were improved by 4- and 2.6-fold, respectively (Table 1). Meanwhile, the corresponding ethyl ester **23** was found to be inactive (IC_{50} > 10 μM), supporting the notion that the COOH moiety with hydrogen bonding donor feature was beneficial.

Moving the electron donating methyl residue in *ortho* position at the benzyloxy moiety to *para* position resulted in a steric hindrance and caused a loss of bioactivity (**34**). When the methyl residue was replaced by an electron-withdrawing chlorine (**36**) or trifluoromethyl (**37**), the potency in cell-based assays slightly improved (IC_{50} = 0.77–1.4 μM) but in the cell-free assay the activity was impaired (IC_{50} = 5.9–6.1 μM). Thus, the cell-free data imply that replacement of an electron donor group in *ortho* position to an electron withdrawing group causes loss of activity, most probably by affecting the π-π interaction capability of the attached phenyl

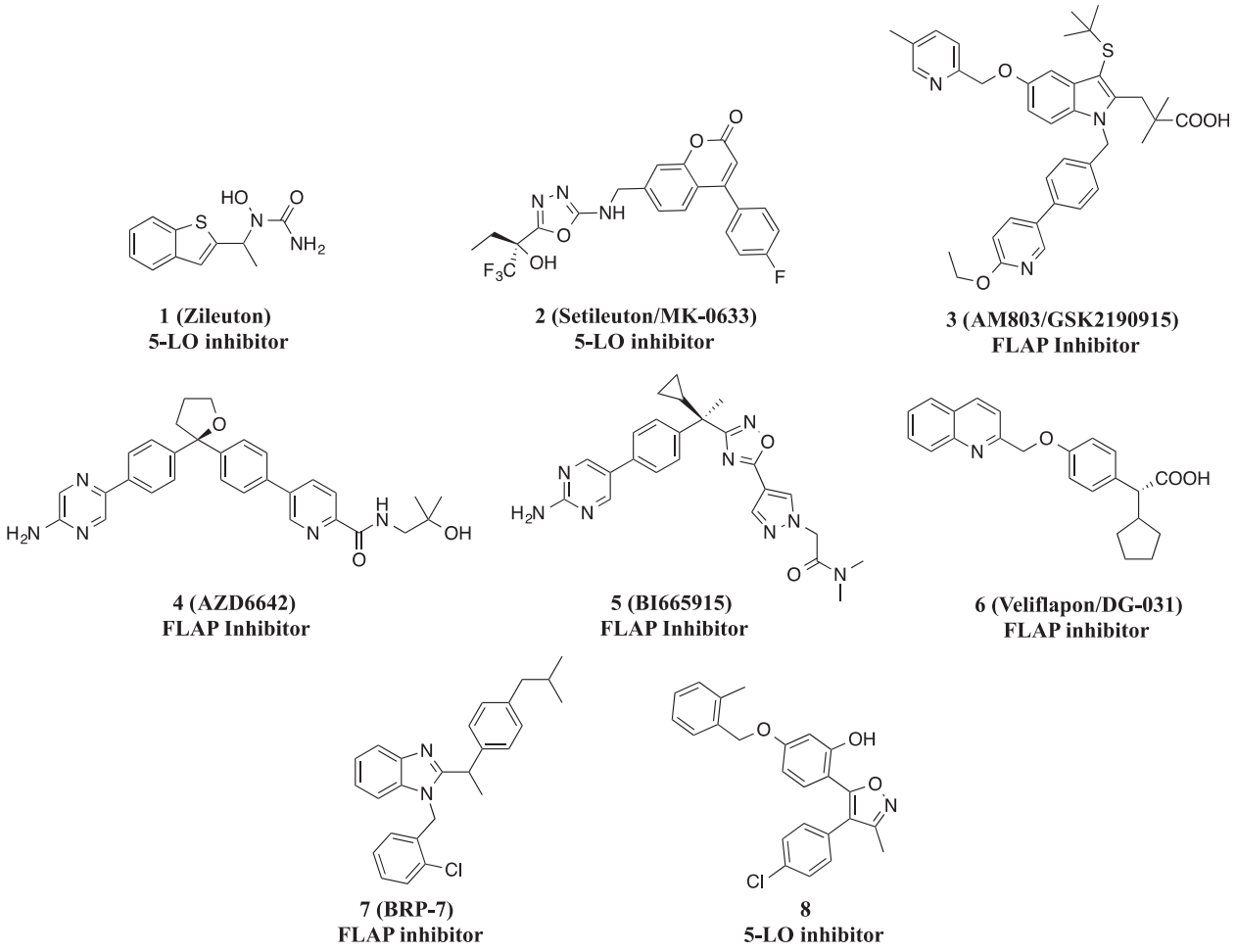


Fig. 1. Chemical structures of LT biosynthesis inhibitors.

group with His372. On the other hand, substitution of the benzyloxy group with fluorine in *meta* position (**33**) was tolerated and slightly increased the bioactivity in both assays, whereas with cyano in this position (**35**), the efficiency clearly dropped ($IC_{50} = 4.1$ and 16.3 μM in cell-based and cell-free test systems, respectively). Replacing the benzyloxy moiety by pyridine-2-yl-methyloxy (**38**) further reduced the 5-LO inhibitory potency in both assay types, similar to 3-cyano derivative **35**. Again, ester analogues of active **33** and **34** abolished the bioactivity (i.e., **24** and **25**), thus highlighting the free acidic carboxyl group as structural determinant (Table 1).

We also investigated the effects of introducing voluminous heteroaromatic groups into the molecule. Intriguingly, a substantial gain of potency in intact cells was obtained when the benzyl was exchanged by a quinolinyl-methyl (**39**) or benzothiazolyl-methyl (**40**) residues with IC_{50} = 0.24 μM, each. Therefore, the compounds **39** and **40** outperformed the reference drug zileuton (**1**) in cell-based assay for which the IC_{50} was determined as 0.91 μM in agreement with the literature [4]. Note that in the cell-free assay **39** and **40** were more than 30-fold less potent ($IC_{50} = 8$ and > 10 μM, respectively), implying that the compounds may target other components than 5-LO in intact cells leading to reduced 5-LO product synthesis. In fact, the 5-LO reference inhibitor **1** was also found equally effective in the cell free assay (IC_{50} = 0.58 μM, Table 1).

Taken together, replacing the 3-methyl moiety at the central isoxazole by a carboxyl group leads to a core structure for potent inhibitors of cellular 5-LO product synthesis. The nature of substituents at the C5 of isoxazole determines the potencies, thus indicating that substitution pattern of the 4-benzyloxyphenyl group is crucial for 5-LO inhibitory activity. Apparently, a consistent SAR for direct 5-LO inhibition is not obvious since small structural differences on the scaffold were not tolerated. Our data underline the importance of the 4-benzyloxyphenyl moiety for directly interacting with 5-LO in this structural context. However, it should be kept in mind that almost all isoxazole-3-carboxylic acid derivatives were markedly active in the 'FLAP-dependent' cell-based assays (Table 1). Because several derivatives of this compound class (e.g., **34**, **36**, **37**, **39** and **40**) potently suppressed cellular 5-LO product formation (IC_{50} = 0.24–1.4 μM) without significantly affecting 5-LO in the cell-free assay ($IC_{50} \geq 5.9$ μM), we speculate that these derivatives primarily act at other targets than 5-LO, presumably on FLAP.

2.4. Docking studies and MD simulations of *39* with FLAP

To support the idea that **39** and **40** may act as FLAP inhibitor, we performed docking studies of a library of compounds including these active molecules, inactive compounds **15**, **16**, **23**–**25** and a set of 1911 decoy compounds as detailed in the Supporting information (Table S1). Specifically, we wondered whether compounds **39** and **40** could show better binding scores with respect to inactive compounds (**15**, **16**, **23**–**25**) in a distribution of binding scores generated with decoy compounds.

All of the compounds were docked to the site located between

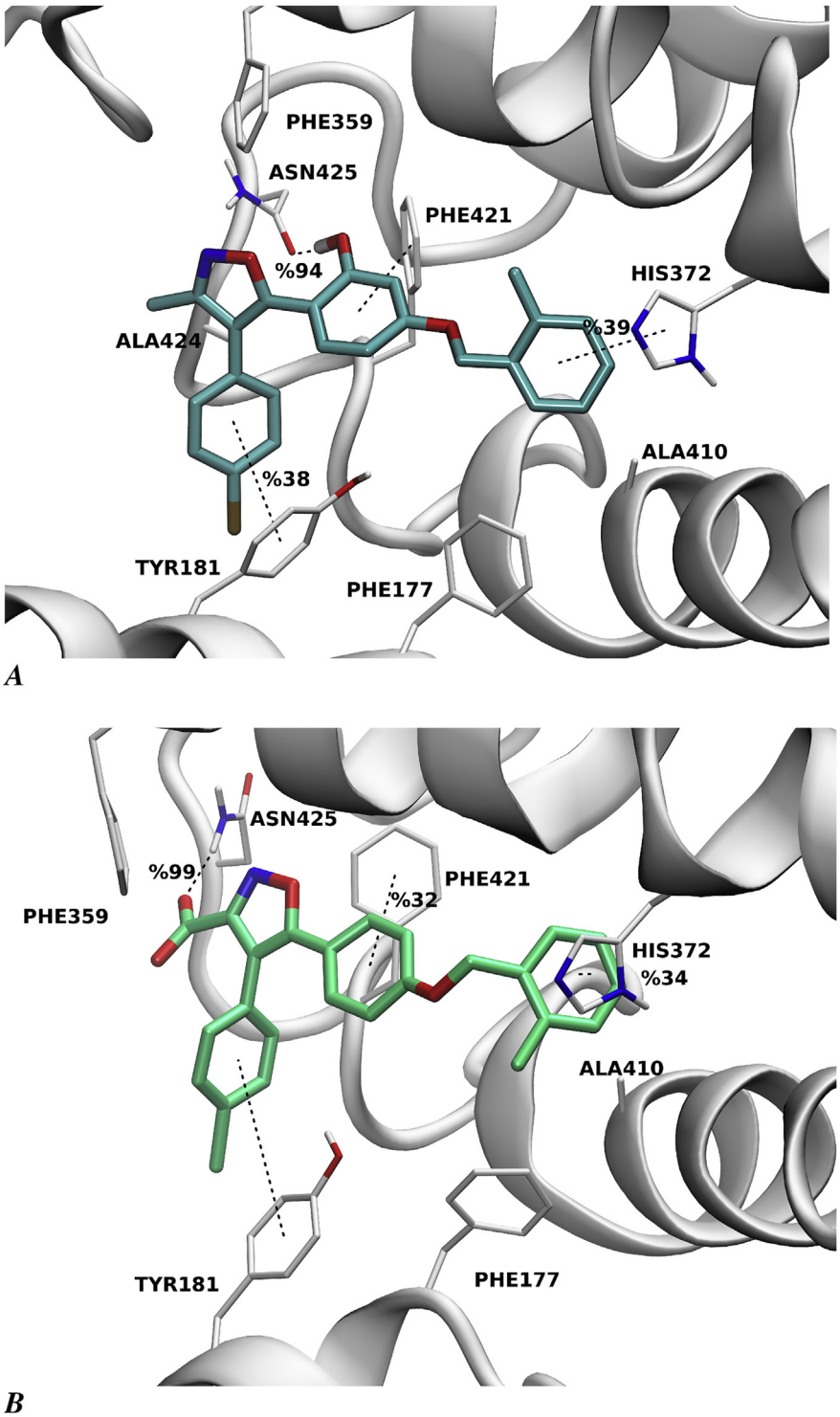
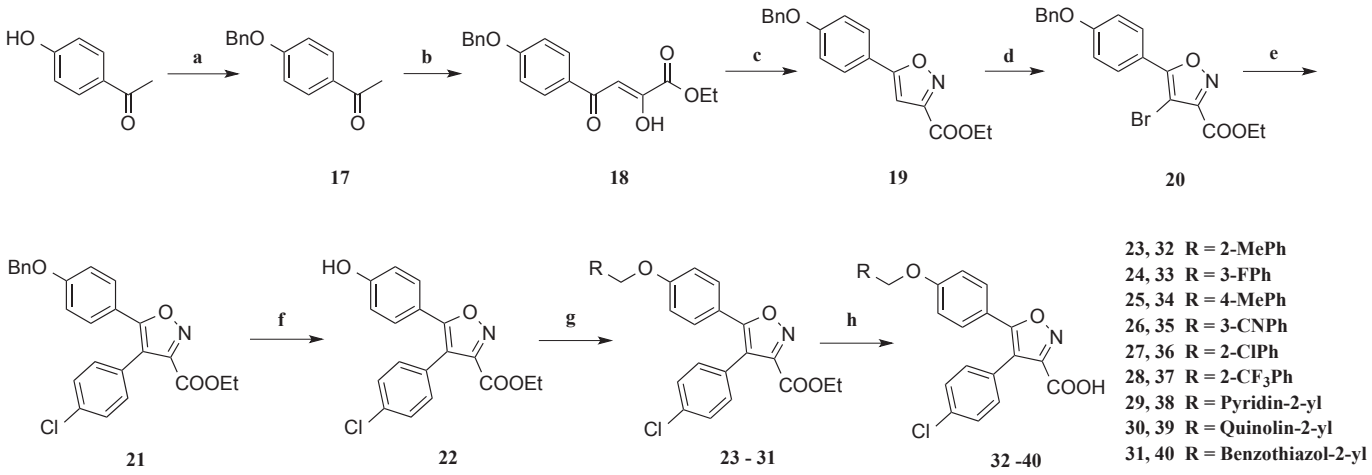


Fig. 2. Binding mode analysis of 3-methylisoxazole derivative **8** (A), and isoxazole-3-carboxylic acid derivative **32** (B), depicted together with interacting 5-LO (PDB code 3O8Y) residues. Main interactions are represented schematically with their occupancies calculated in the time window 0–10 ns.

the chains B and C with the key residues B-Phe123, B-Leu120, B-Ile119, B-Lys116, B-Phe114, B-Ile113, B-Tyr112, B-Thr66, B-Asp63, C-Val61, C-His28, C-Ala27, C-Phe25 and C-Asn23 as we reported previously [29]. As a result, we observed that the binding scores (Emodel score) of **39** and **40** ranked among the top 2.13% and 4.11% of the distribution, respectively. In contrast, binding scores of the inactive compounds started ranking after 18% of the distribution. Hence, docking studies supported the hypothesis that quinoline derivative **39** and its benzothiazole analogue **40** are indeed FLAP inhibitors. Taking into account the docking calculations reported for FLAP before [40–42] and shown in this study, the binding specificity of **39** was conferred by the H-bond with Lys116 (Fig. S4). Moreover, our suggested pose for **39** adopts the equivalent spatial disposition of the co-crystallized inhibitor **41** (MK-591),



Scheme 2. Synthesis of 4,5-diarylisoxazol-3-carboxylic acids. Reagents and conditions: (a) BnBr, K_2CO_3, MeCN, reflux; (b) $(CO_2Et)_2$, NaOEt, EtOH, rt; (c) $NH_2OH \cdot HCl$, EtOH, reflux; (d) NBS, CAN, AcCN; (e) 4-chlorophenylboronic acid, $Pd(PPh_3)_2Cl_2$, $NaHCO_3$, H_2O, DMF; (f) Pd/C, H_2, MeOH:THF (1:1); (g) appropriate benzyl halide, 2-(chloromethyl)pyridine, 2-(chloromethyl)quinoline or 2-(chloromethyl)benzothiazole, K_2CO_3, KI, MeCN, reflux; (h) LiOH, THF, H_2O.

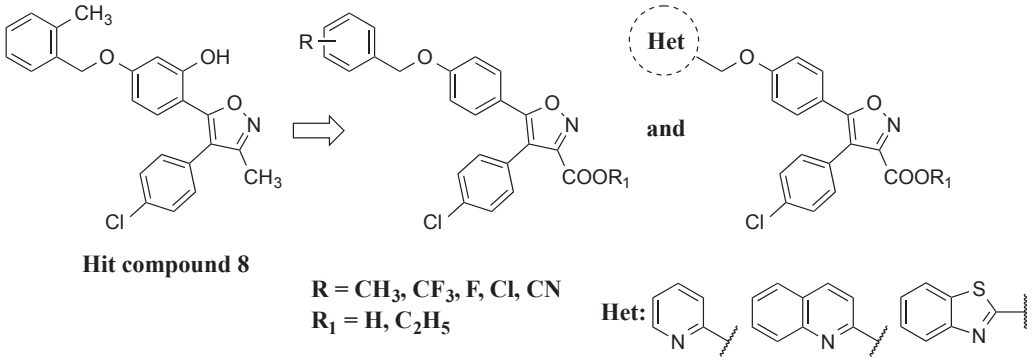


Fig. 3. Major structural modifications of hit compound **8**.

maintaining the same interaction network that accounts for a high affinity binding mode to FLAP (Figs. S3–S4).

Accordingly, to further investigate the binding mode of **39** in comparison with **41** (MK591) to FLAP taking into account potential ligand-induced conformational changes, we performed MD simulations using the 3D structure of FLAP (PDB code 2Q7M) in complex with these docked ligands (see Supporting information, Figs. S3–4) [39]. Since the available FLAP x-ray structure has a low resolution (4.2 Å) and does not include membrane coordinates, we analyzed the binding mode of FLAP/**41** and FLAP/**39** complexes by means of MD simulations including membrane insertion (taken from OPM Database [43]) in the simulation systems (Fig. 4A–B). By inclusion of the membrane bilayer, it was possible to observe its effect on the binding in which the adopted techniques have been widely used for the analysis of other membrane proteins [44]. As a result, the binding of **39** to FLAP is characterized by several interactions, but the most stable ones derived from the MD simulation data shows: (i) the carboxylic acid group makes polar interactions mainly with B-Lys116 (occupancy, 42%) and C-His28 (occupancy, 36%); (ii) the quinoline group engages a π-π interaction with B-Tyr112 (occupancy, 38%) and makes hydrophobic contacts to B-Thr66 (occupancy, 28%) (Fig. 4B).

3. Conclusions

We have designed, synthesized and biologically evaluated a set of new isoxazole derivatives based on the structure of hit **8** that we previously identified as an inhibitor of 5-LO product synthesis [29]. We demonstrated that although the activity of all derivatives in cell-based assays were quite pronounced, the different substitutions on the benzyloxyphenyl arm at position C5 of the isoxazole caused a great discrepancy for inhibition of 5-LO product synthesis among this class of compounds, where only **32**–**33** exhibited potent direct 5-LO inhibitory activity (IC_{50} 5-LO = 0.48 and 0.38 μM, respectively), while other derivatives were efficient inhibitors of cellular 5-LO product synthesis, seemingly by acting on FLAP (e.g, **34**, **36**, **37**, **39**, **40**; IC_{50} cell-based = 0.24–1.4 μM). By means of docking studies and MD simulations with 5-LO and FLAP, satisfactory explanations of the putative binding mode for these isoxazole-based 5-LO inhibitors were provided, each, demonstrating the preservation of the important ligand-protein interactions as seen in crystal structures. Based on our results, we can speculate that the ability of these compounds to interfere with LT biosynthesis is either caused by 5-LO or FLAP inhibition according to the superior activity in cell-free or cell-based assays, respectively.

In conclusion, our biological results disclosed two benchmark compounds **39** and **40** with potent inhibitory activity against cellular 5-LO product synthesis (IC_{50} = 0.24 μM, each) most likely due to interference with FLAP but also with weak 5-LO suppression ($IC_{50} \geq 8.0$ μM). These isoxazole-3-carboxylic acids may be suitable for further development of therapeutics in diseases requiring anti-LT therapy such as respiratory diseases, cardiovascular diseases,

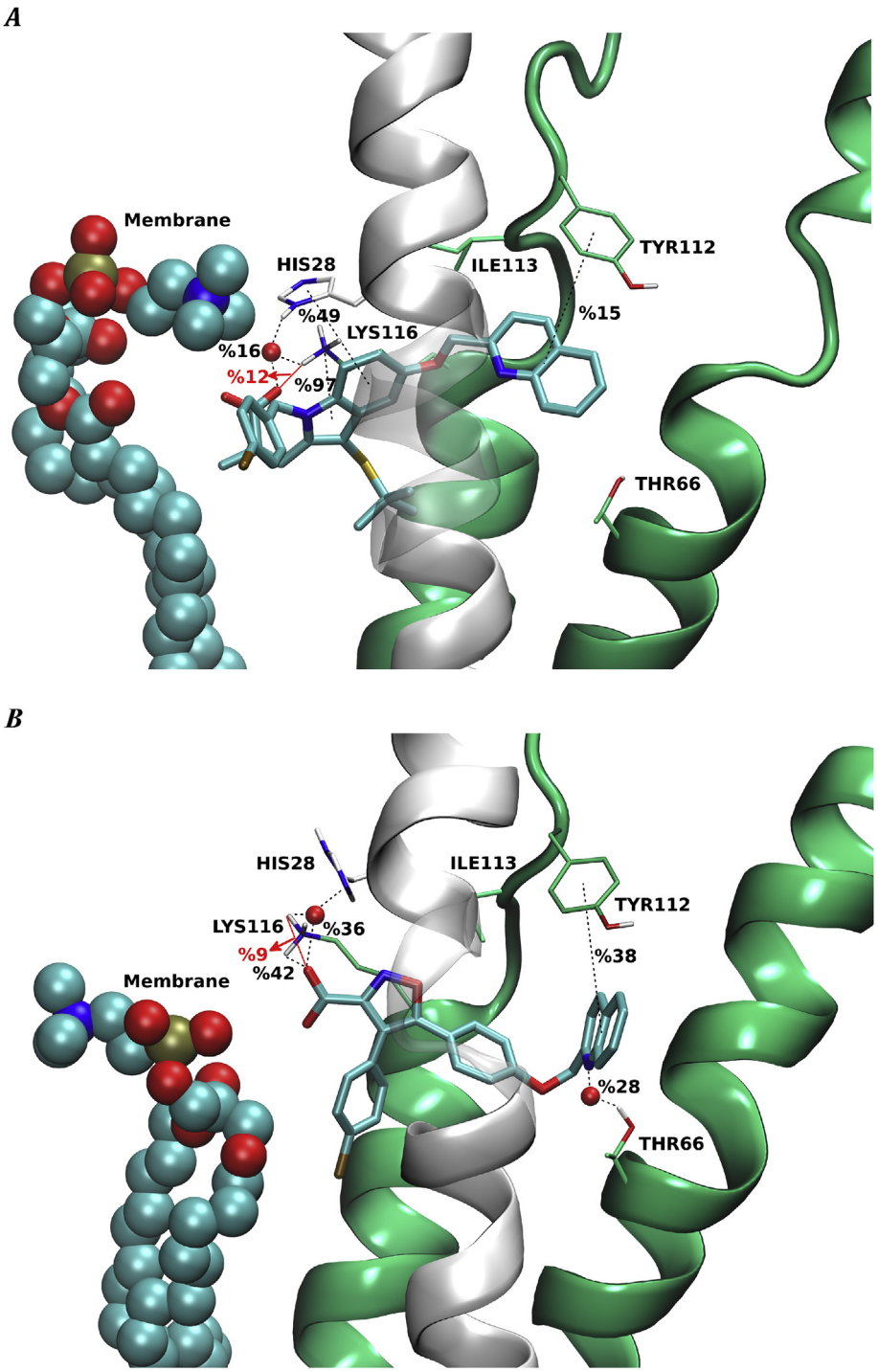


Fig. 4. X-ray binding mode analysis of ligand **41** (MK591) (A) and **39** (B) during interaction with FLAP (PDB code 2Q7M) considering membrane residues. Main interactions are represented schematically with their occupancies calculated in the time window 0–10 ns.

allergic and autoimmune diseases as well as cancer. An ongoing comprehensive pharmacological study, beyond the scope of this work, using various cell-based and cell-free assays combined with microscopic techniques (e.g. proximity ligation assay), may provide additional evidence for **39** acting on FLAP in the future.

4. Experimental section

4.1. Chemistry

Extensive experimental information on synthetic procedures can be found in Supporting information. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 on a Varian Mercury 400 MHz or Bruker Ultrashield 300 MHz High Performance Digital FT-NMR spectrometer using tetramethylsilane as the internal standard. All

chemical shifts were recorded as δ (ppm). High resolution mass spectra data (HRMS) were collected using a Waters LCT Premier XE Mass Spectrometer (high sensitivity orthogonal acceleration time-of-flight instrument) operating in ESI (+) method, also coupled to an AQUITY Ultra Performance Liquid Chromatography system (Waters Corporation, Milford, MA, USA) using a UV detector monitoring at 254 nm with a Aquity BEH C18 column (2.1 × 100 mm, 1.7 μm, 0.3 mL/min flow rate), using a gradient of CH_3CN/H_2O (1%–90%) containing 1% formic acid. All tested compounds were determined to be ≥ 95% pure using the analytical method described above based on the peak area percentage. Elemental analyses were taken on a Leco 932 CHNS analyzer. Melting points were determined with an SMP-II Digital Melting Point Apparatus and are uncorrected (Schorpp Geraetetechnik, Germany). Flash chromatography was performed with a Combiflash® Rf automated flash chromatography system with RediSep columns (Teledyne-Isco, Lincoln, NE, USA) using *n*-hexane–EtOAc or DCM–MeOH solvent gradient.

*4.1.1. Synthesis of 3-methylisoxazole derivatives (**15**–**16**)*

A solution of KI (1.8 mmol) and iodine (0.5 mmol) in H_2O (4 mL) was added to a solution of **13**–**14** (0.5 mmol) and $NaHCO_3$ (2.1 mmol) in THF (6 mL) and water (6 mL) in a reaction flask covered by aluminum foil. After the reaction mixture was heated at reflux for 5 h, saturated aqueous sodium bisulfite solution (5 mL) was added, and this mixture was extracted with EtOAc (2 × 50 mL). The combined organic extracts were dried over anhydrous $MgSO_4$, the solvent was removed in vacuo, and the residue was purified by automated flash column chromatography using hexane-ethyl acetate gradient as eluent to yield **15**–**16.**

*4.1.1.1. 4-(4-Chlorophenyl)-5-{4-[(2-methylphenyl)methoxy]phenyl}-3-methylisoxazole (**15**).* Purified by flash column chromatography (0% → 30% EtOAc in hexane). Yield 63%; mp 155.8–156.1 °C. ^{1}H NMR ($CDCl_3$): δ 2.22 (3H, s), 2.36 (3H, s), 5.04 (2H, s), 6.94 (2H, d, *J* = 8.8 Hz), 7.22–7.27 (5H, m), 7.37 (1H, m), 7.42 (2H, d, *J* = 8.8 Hz), 7.46 (2H, d, *J* = 9.2 Hz). ^{13}C NMR ($CDCl_3$): δ 10.62, 18.89, 68.64, 113.79, 114.96, 120.48, 126.10, 128.44, 128.48, 128.62, 129.37, 129.40, 130.49, 131.26, 134.05, 134.20, 136.70, 159.80, 160.09, 164.59. Anal. Calcd for $C_{24}H_{20}ClNO_2$: C, 73.94; H, 5.17; N, 3.59; found: C, 73.88; H, 5.11; N, 3.72. HRMS *(m/z):* $[M+H]^+$ *calcd* for $C_{24}H_{21}ClNO_2$ 391.1339, *found* 391.1336.

*4.1.1.2. 4-(4-Chlorophenyl)-5-{2-methoxy-4-[(2-methylphenyl) methoxy]phenyl}-3-methylisoxazole (**16**).* Purified by flash column chromatography (0% → 30% EtOAc in hexane). Yield 23%; mp 131.8–132.2 °C. ^{1}H NMR ($CDCl_3$): δ 2.33 (3H, s), 2.39 (3H, s), 3.38 (3H, s), 5.05 (2H, s), 6.48 (1H, d, *J* = 2.0 Hz), 6.63 (1H, dd, *J* = 2.0, 8.8 Hz), 7.12 (2H, d, *J* = 8.4 Hz), 7.21–7.28 (3H, m), 7.31 (2H, d, *J* = 8.4 Hz), 7.38–7.41 (2H, m). ^{13}C NMR ($CDCl_3$): δ 11.25, 19.15, 29.93, 55.12, 69.04, 99.90, 105.89, 110.34, 116.24, 126.36, 128.77, 128.83, 129.08, 130.19, 130.73, 130.76, 131.82, 133.18, 134.38, 137.06, 158.05, 159.10, 162.18, 163.99. Anal. Calcd for $C_{25}H_{22}ClNO_3 \cdot 0.4H_2O$: C, 70.30; H, 5.38; N, 3.28; found: C, 70.30; H, 5.46; N, 3.18. HRMS *(m/z):* $[M+H]^+$ *calcd* for $C_{25}H_{23}ClNO_3$ 420.1366, *found* 420.1364.

*4.1.2. Synthesis of title isoxazol-3-carboxylic acid derivatives (**32**–**40**)*

Ester intermediates **23**–**31** (0.003 mol) and lithium hydroxide (0.008 mol) in THF/water mixture (1:1) were stirred at room temperature for 4 h. After the reaction is complete, the reaction mixture was diluted with water, acidified with 2N HCl and the precipitate was filtered and recrystallized from the appropriate solvent or purified by automated flash chromatography.

*4.1.2.1. 4-(4-Chlorophenyl)-5-{4-[(2-methylbenzyl)oxy]phenyl}isoxazol-3-carboxylic acid (**32**).* Purified by recrystallization from methanol. Yield 49.4%; mp 188.7–189.8 °C. ^{1}H NMR (DMSO-d_6): δ 2.30 (3H, s), 5.10 (2H, s), 7.10 (2H, d, *J* = 8.0 Hz), 7.18–7.25 (3H, m), 7.37–7.41 (5H, m), 7.51 (2H, d, *J* = 8.0 Hz). ^{13}C NMR (DMSO-d_6): δ 18.41, 68.11, 113.81, 115.34, 118.84, 125.77, 128.19, 128.23, 128.41, 128.58, 128.65, 130.14, 132.08, 133.10, 134.37, 136.65, 155.92, 160.14, 160.87, 166.40. Anal. Calcd for $C_{24}H_{18}ClNO_4 \cdot 0.5MeOH$ C, 67.51; H, 4.62; N, 3.21; found: C, 67.38; H, 4.81; N, 3.37. HRMS *(m/z):* $[M+H]^+$ *calcd* for $C_{24}H_{19}ClNO_4$ 420.1003, *found* 420.0984.

*4.1.2.2. 4-(4-Chlorophenyl)-5-{4-[(3-fluorobenzyl)oxy]phenyl}isoxazol-3-carboxylic acid (**33**).* Purified by recrystallization from isopropanol. Yield 65%; mp 190.4–191.1 °C. ^{1}H NMR (DMSO-d_6): δ 5.15 (2H, s), 7.09 (2H, d, *J* = 8.8 Hz), 7.14–7.19 (1H, m), 7.26–7.28 (2H, m), 7.37–7.46 (5H, m), 7.50 (2H, 2H, d, *J* = 8.8 Hz). ^{13}C NMR (DMSO-d_6): δ 68.46, 113.81, 114.25 (d, *J* = 22.1 Hz), 114.65 (d, *J* = 20.6 Hz), 115.37, 118.96, 123.53 (d, *J* = 3.1 Hz), 128.12, 128.38, 128.56, 130.42 (d, *J* = 8.4 Hz), 131.99, 133.05, 139.36 (d, *J* = 7.6 Hz), 155.88, 159.74, 160.78, 162.10 (d, *J* = 242.3 Hz), 166.28. Anal. Calcd for $C_{23}H_{15}ClFNO_4$ C, 65.18; H, 3.57; N, 3.30; found: C, 64.79; H, 3.84; N, 3.49. HRMS *(m/z):* $[M+H]^+$ *calcd* for $C_{23}H_{16}ClFNO_4$ 424.0752, *found* 424.0734.

*4.1.2.3. 4-(4-Chlorophenyl)-5-{4-[(4-methylbenzyl)oxy]phenyl}isoxazol-3-carboxylic acid (**34**).* Purified by recrystallization from methanol. Yield 88%; mp 185.9–186.6 °C. ^{1}H NMR (DMSO-d_6): δ 2.29 (3H, s), 5.06 (2H, s), 7.05 (2H, d, *J* = 8.4 Hz), 7.18 (2H, d, *J* = 8.0 Hz), 7.30 (2H, d, *J* = 8.0 Hz), 7.36–7.39 (4H, m), 7.49 (2H, d, *J* = 8.4 Hz). ^{13}C NMR (DMSO-d_6): δ 20.75, 69.30, 113.79, 115.40, 118.76, 127.91, 128.22, 128.39, 128.64, 129.99, 132.07, 133.09, 133.43, 137.27, 155.93, 160.06, 160.88, 166.40. Anal. Calcd for $C_{24}H_{18}ClNO_4$ C, 68.66; H, 4.32; N, 3.34; found: C, 68.64; H, 3.98; N, 3.50. HRMS *(m/z):* $[M+H]^+$ *calcd* for $C_{24}H_{19}ClNO_4$ 420.1003, *found* 420.0982.

*4.1.2.4. 4-(4-Chlorophenyl)-5-{4-[(3-cyanobenzyl)oxy]phenyl}isoxazol-3-carboxylic acid (**35**).* Purified by recrystallization from methanol. Yield 48%; mp 180.5–181.0 °C. ^{1}H NMR (DMSO-d_6): δ 5.20 (2H, s), 7.10 (2H, d, *J* = 8.8 Hz), 7.38–7.42 (4H, m), 7.50 (2H, d, *J* = 8.8 Hz), 7.61 (1H, t, *J* = 8.0 Hz), 7.77–7.82 (2H, m), 7.91 (1H, s). ^{13}C NMR (DMSO-d_6): δ 68.13, 111.42, 113.88, 115.38, 118.53, 119.05, 128.12, 128.42, 128.59, 129.70, 131.10, 131.72, 132.01, 132.45, 133.06, 138.17, 155.84, 159.61, 160.79, 166.28. Anal. Calcd for $C_{24}H_{15}ClN_2O_4$ C, 66.91; H, 3.51; N, 6.50; found: C, 66.57; H, 3.73; N, 6.36. HRMS *(m/z):* $[M+H]^+$ *calcd* for $C_{24}H_{16}ClN_2O_4$ 431.0799, *found* 431.0782.

*4.1.2.5. 4-(4-Chlorophenyl)-5-{4-[(2-chlorobenzyl)oxy]phenyl}isoxazol-3-carboxylic acid (**36**).* Purified by recrystallization from methanol. Yield 93%; mp 128.5 °C. ^{1}H NMR (DMSO-d_6): δ 5.18 (2H, s), 7.13 (2H, d, *J* = 8.8 Hz), 7.38–7.43 (6H, m), 7.49–7.53 (3H, m), 7.58–7.60 (1H, m). ^{13}C NMR (DMSO-d_6): δ 67.78, 114.61, 116.04, 119.82, 128.08, 128.88, 129.19, 129.36, 130.13, 130.77, 131.03, 132.78, 133.48, 133.81, 134.45, 156.64, 160.55, 161.56, 167.04. Anal. Calcd for $C_{23}H_{15}Cl_2NO_4$ C, 62.74; H, 3.43; N, 3.18; found: C, 62.99; H, 3.53; N, 3.51. HRMS *(m/z):* $[M+H]^+$ *calcd* for $C_{24}H_{16}Cl_2NO_4$ 440.0456, *found* 440.0446.

*4.1.2.6. 4-(4-Chlorophenyl)-5-{4-[(2-trifluoromethylbenzyl)oxy] phenyl}isoxazol-3-carboxylic acid (**37**).* Purified by recrystallization from methanol. Yield 70%; mp 156.3 °C. ^{1}H NMR ($CDCl_3$): δ 5.28 (2H, s), 6.92–6.94 (2H, d, *J* = 9.2 Hz), 7.28–7.30 (2H, d, *J* = 8.4 Hz), 7.39–7.46 (5H, m), 7.55–7.59 (1H, t, *J* = 7.6 Hz), 7.68–7.71 (2H, t, *J* = 7.4 Hz). ^{13}C NMR (DMSO-d_6): δ 66.35, 113.89, 115.23, 119.16, 124.16 (q, *J* = 272.1 Hz), 126.14 (q, *J* = 5.3 Hz), 126.96 (q, *J* = 30.4 Hz), 128.10, 128.45, 128.60, 128.88, 130.69, 132.02, 132.78, 133.06, 134.20,

155.86, 159.63, 160.79, 166.24. Anal. Calcd for $C_{24}H_{15}ClF_3NO_4 \cdot 0.4CH_3OH$ C, 60.22; H, 3.44; N, 2.88; found: C, 60.16; H, 3.54; N, 3.20. HRMS *(m/z):* $[M+H]^+$ *calcd* for $C_{24}H_{16}ClF_3NO_4$ 474.0720, *found* 474.0730.

*4.1.2.7. 4-(4-Chlorophenyl)-5-[4-(pyridin-2-ylmethoxy)phenyl]isoxazol-3-carboxylic acid (**38**).* Purified by flash column chromatography (0% → 20% MeOH in DCM) and recrystallized from methanol. Yield 32%; mp 176.8 °C. ^{1}H NMR (DMSO-d_6): δ 5.20 (2H, s), 7.10 (2H, d, J = 8.8 Hz), 7.34–7.52 (8H, m), 7.82–7.86 (1H, m), 8.54 (1H, d, J = 4.8 Hz). ^{13}C NMR (DMSO-d_6): δ 70.39, 113.89, 115.41, 119.04, 121.82, 123.10, 128.19, 128.46, 128.65, 132.08, 133.11, 137.04, 149.14, 155.93, 156.07, 159.85, 160.87, 166.33. Anal. Calcd for $C_{22}H_{15}ClN_2O_4 \cdot 0.3CH_3OH$ C, 64.32; H, 3.92; N, 6.73; found: C, 64.35; H, 3.96; N, 6.67. HRMS *(m/z):* $[M+H]^+$ *calcd* for $C_{22}H_{16}ClN_2O_4$ 407.0799, *found* 407.0782.

*4.1.2.8. 4-(4-Chlorophenyl)-5-[4-(quinolin-2-ylmethoxy)phenyl]isoxazol-3-carboxylic acid (**39**).* Purified by recrystallization from isopropanol. Yield 94%; mp 208.4 °C. ^{1}H NMR (DMSO-d_6): δ 5.40 (2H, s), 7.15 (2H, d, J = 8.8 Hz), 7.38–7.42 (4H, m), 7.50 (2H, d, J = 8.8 Hz), 7.61–7.67 (2H, m), 7.76–7.80 (1H, m), 7.99 (2H, m), 8.42 (1H, d, J = 8.0 Hz). ^{13}C NMR (DMSO-d_6): δ 70.84, 113.84, 115.42, 119.05, 119.52, 126.59, 127.13, 127.88, 128.09, 128.42, 128.47, 128.58, 129.85, 131.99, 133.03, 137.05, 146.86, 155.84, 156.87, 159.78, 160.77, 166.23. Anal. Calcd for $C_{26}H_{17}ClN_2O_4 \cdot 1.0\ HOCH(CH_3)_2$ C, 67.93; H, 4.23; N, 5.83; found: C, 67.62; H, 4.26; N, 5.99. HRMS *(m/z):* $[M+H]^+$ *calcd* for $C_{26}H_{18}ClN_2O_4$ 457.0955, *found* 457.0954.

*4.1.2.9. 4-(4-Chlorophenyl)-5-[4-(benzothiazol-2-ylmethoxy)phenyl] isoxazol-3-carboxylic acid (**40**).* Purified by recrystallization from methanol. Yield 89%; mp 196.5 °C. ^{1}H NMR (DMSO-d_6): δ 5.65 (2H, s), 7.18 (2H, d, J = 9.2 Hz), 7.38–7.56 (8H, m), 8.02 (1H, d, J = 8.4 Hz), 8.12 (1H, d, J = 7.6 Hz). ^{13}C NMR (DMSO-d_6): δ 67.11, 114.02, 115.53, 119.64, 122.35, 122.69, 125.34, 126.30, 128.03, 128.47, 128.58, 131.98, 133.06, 134.34, 152.37, 155.89, 159.02, 160.75, 166.08, 167.51. Anal. Calcd for $C_{24}H_{15}ClN_2O_4S \cdot 0.5CH_3OH$ C, 61.44; H, 3.38; N, 5.85; S, 6.70 found: C, 61.49; H, 3.36; N, 6.20; S, 6.94. HRMS *(m/z):* $[M+H]^+$ *calcd* for $C_{24}H_{16}ClN_2O_4S$ 463.0519, *found* 463.0516.

4.2. Biological assays

4.2.1. Cells

Neutrophils were isolated from human blood as reported before [45]. Briefly, human peripheral blood was obtained from fastened (12 h) healthy donors with consent that had not taken anti-inflammatory drugs during the last 10 days, by venipuncture in heparinized tubes (16 IE heparin/ml blood; University Hospital Jena, Germany). The blood was centrifuged (4000 × g for 20 min at 20 °C) for preparation of leukocyte concentrates. Leukocyte concentrates were then subjected to dextran sedimentation and centrifugation on Nycoprep cushions. Contaminating erythrocytes of pelleted neutrophils were lysed by hypotonic lysis using water. Neutrophils were washed twice in ice-cold PBS (purity > 96–97%) and finally resuspended in PBS pH 7.4 containing 1 mg/ml glucose and 1 mM $CaCl_2$ (PGC buffer).

For analysis of acute cytotoxicity of the compounds during pre-incubation periods (30 min at 37 °C), cellular integrity of neutrophils was analyzed by trypan blue exclusion with a Vi-cell counter (Beckmann Coulter GmbH, Krefeld). None of the compounds caused significant loss of neutrophil viability within 30 min (data not shown).

4.2.2. Determination of 5-LO products in intact cells

For determination of LO products in intact cells, neutrophils (5×10^6) were resuspended in 1 ml PGC buffer, preincubated for 15 min at 37 °C with test compounds or vehicle (0.1% DMSO) and Ca^{2+}-ionophore A23187 (2.5 μM) was added. After 10 min at 37 °C the reaction was stopped on ice by addition of 1 ml of methanol. 30 μl 1 N HCl and 500 μl PBS, and 200 ng prostaglandin (PG)B_1 were added and the samples were subjected to solid phase extraction on C18-columns (100 mg, UCT, Bristol, PA, USA). 5-LO products (LTB_4 and its trans-isomers, and 5-H(P)ETE) were analyzed by RP-HPLC and quantities calculated on the basis of the internal standard PGB_1. Cys-LTs C_4, D_4 and E_4 were not detected (amounts were below detection limit), and oxidation products of LTB_4 were not determined.

4.2.3. Expression, purification and cell-free activity analysis of human recombinant 5-LO

Escherichia coli BL21 cells were transformed with pT3-5-LO plasmid, and recombinant 5-LO protein was expressed at 27 °C as described [46]. Cells, resuspendend in 50 mM triethanolamine/HCl pH 8.0, 5 mM EDTA, soybean trypsin inhibitor (60 μg/ml), 1 mM phenylmethanesulphonyl fluoride, and lysozyme (500 μg/ml), were homogenized by sonication (3 × 15 s). After centrifugation at 40,000 × g for 20 min at 4 °C, the supernatant was applied to an ATP-agarose column to partially purify 5-LO as described previously [46].

Aliquots of semi-purified 5-LO were diluted with ice-cold PBS containing 1 mM EDTA, and 1 mM ATP was added. Samples were pre-incubated with the test compounds or vehicle (0.1% DMSO) as indicated. After 15 min at 4 °C, samples were pre-warmed for 30 s at 37 °C, and 2 mM $CaCl_2$ plus 20 μM AA was added to initiate 5-LO product formation. After 10 min at 37 °C, the reaction was stopped by addition of 1 ml ice-cold methanol, and the formed metabolites were analyzed by RP-HPLC as described [47]. 5-LO products include the all-trans isomers of LTB_4 and 5-H(P)ETE.

4.3. Statistics

Data are expressed as mean ± S.E.M. of single determinations performed in three or four independent experiments at different days. IC_{50} values were calculated by nonlinear regression using SigmaPlot 9.0 (Systat Software Inc., San Jose, USA) one site binding competition. Statistical evaluation of the data was performed by one-way ANOVA followed by a Bonferroni or Tukey-Kramer post-hoc test for multiple comparisons respectively. A p value < 0.05 (*) was considered significant.

4.4. Computational methods

All the methodologies and parameters used in this study are reported in Supplementary information.

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Appendix A. Supplementary information

Supplementary information related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.02.027.

References

[1] J.Z. Haeggstrom, C.D. Funk, Lipoxygenase and leukotriene pathways: biochemistry, biology, and roles in disease, Chem. Rev. 111 (2011)

5866–5898.
[2] O. Radmark, B. Samuelsson, Regulation of the activity of 5-lipoxygenase, a key enzyme in leukotriene biosynthesis, Biochem. Biophys. Res. Commun. 396 (2010) 105–110.
[3] O. Radmark, O. Werz, D. Steinhilber, B. Samuelsson, 5-lipoxygenase, a key enzyme for leukotriene biosynthesis in health and disease, Biochim. Biophys. Acta 1851 (2015) 331–339.
[4] O. Werz, 5-lipoxygenase: cellular biology and molecular pharmacology, Curr. Drug Targets Inflamm. Allergy 1 (2002) 23–44.
[5] J. Gerstmeier, C. Weinigel, S. Rummler, O. Radmark, O. Werz, U. Garscha, Time-resolved in situ assembly of the leukotriene-synthetic 5-lipoxygenase/5-lipoxygenase-activating protein complex in blood leukocytes, FASEB J. (2015), http://dx.doi.org/10.1096/fj.15-278010. Epub.
[6] S.E. Wenzel, A.K. Kamada, Zileuton: the first 5-lipoxygenase inhibitor for the treatment of asthma, Ann. Pharmacother. 30 (1996) 858–864.
[7] J.A. Bernstein, N. Liu, B.A. Knorr, S.S. Smugar, W.D. Hanley, T.F. Reiss, S. Greenberg, MK-0633, a potent 5-lipoxygenase inhibitor, in chronic obstructive pulmonary disease, Respir. Med. 105 (2011) 392–401.
[8] Y. Ducharme, M. Blouin, C. Brideau, A. Chateauneuf, Y. Gareau, E.L. Grimm, H. Juteau, S. Laliberte, B. MacKay, F. Masse, M. Ouellet, M. Salem, A. Styhler, R.W. Friesen, The discovery of setileuton, a potent and selective 5-lipoxygenase inhibitor, ACS Med. Chem. Lett. 1 (2010) 170–174.
[9] Y.S. Wasfi, C. Villaran, B. de Tilleghem Cle, S.S. Smugar, W.D. Hanley, T.F. Reiss, B.A. Knorr, The efficacy and tolerability of MK-0633, a 5-lipoxygenase inhibitor, in chronic asthma, Respir. Med. 106 (2012) 34–46.
[10] R. Chaudhuri, V. Norris, K. Kelly, C.Q. Zhu, C. Ambery, J. Lafferty, E. Cameron, N.C. Thomson, Effects of a FLAP inhibitor, GSK2190915, in asthmatics with high sputum neutrophils, Pulm. Pharmacol. Ther. 27 (2014) 62–69.
[11] R.M. Follows, N.G. Snowise, S.Y. Ho, C.L. Ambery, K. Smart, B.A. McQuade, Efficacy, safety and tolerability of GSK2190915, a 5-lipoxygenase activating protein inhibitor, in adults and adolescents with persistent asthma: a randomised dose-ranging study, Respir. Res. 14 (2013) 54.
[12] M. Lemurell, J. Ulander, S. Winiwarter, A. Dahlen, O. Davidsson, H. Emtenas, J. Broddefalk, M. Swanson, D. Hovdal, A.T. Plowright, A. Pettersen, M. Ryden-Landergren, J. Barlind, A. Llinas, M. Herslof, T. Drmota, K. Sigfridsson, S. Moses, C. Whatling, Discovery of AZD6642, an inhibitor of 5-lipoxygenase activating protein (FLAP) for the treatment of inflammatory diseases, J. Med. Chem. 58 (2015) 897–911.
[13] H. Takahashi, D. Riether, A. Bartolozzi, T. Bosanac, V. Berger, R. Binetti, J. Broadwater, Z. Chen, R. Crux, S. De Lombaert, R. Dave, J.A. Dines, T. Fadra-Khan, A. Flegg, M. Garrigou, M.H. Hao, J. Huber, J.M. Hutzler, S. Kerr, A. Kotey, W. Liu, H.Y. Lo, P.L. Loke, P.E. Mahaney, T.M. Morwick, S. Napier, A. Olague, E. Pack, A.K. Padyana, D.S. Thomson, H. Tye, L. Wu, R.M. Zindell, A. Abeywardane, T. Simpson, Synthesis, SAR, and series evolution of novel oxadiazole-containing 5-lipoxygenase activating protein inhibitors: discovery of 2-[4-(3-{(r)-1-[4-(2-amino-pyrimidin-5-yl)-phenyl]-1-cyclopropyl-ethyl}-[1,2,4]oxadiazol-5-yl)-pyrazol-1-yl]-N,N-dimethyl-acetamide (BI 665915), J. Med. Chem. 58 (2015) 1669–1690.
[14] C. Whatling, W. McPheat, M. Herslof, The potential link between atherosclerosis and the 5-lipoxygenase pathway: investigational agents with new implications for the cardiovascular field, Expert. Opin. Investig. Drugs 16 (2007) 1879–1893.
[15] S. Xu, L. Tang, Y. Mi, J. Jiang, M. Zhu, B. Chen, B. Wang, T. Li, C. Xu, J. Wang, X. Lu, W. Ge, L. Wang, J. Huang, Clinical significance of leukotriene b4 and extracellular matrix metalloproteinase inducer in acute coronary syndrome, Clin. Investig. Med. 36 (2013) E282–E289.
[16] M. Back, Inflammatory signaling through leukotriene receptors in atherosclerosis, Curr. Atheroscler. Rep. 10 (2008) 244–251.
[17] R. De Caterina, A. Zampolli, From asthma to atherosclerosis–5-lipoxygenase, leukotrienes, and inflammation, N. Engl. J. Med. 350 (2004) 4–7.
[18] R. Spanbroek, R. Grabner, K. Lotzer, M. Hildner, A. Urbach, K. Ruhling, M.P. Moos, B. Kaiser, T.U. Cohnert, T. Wahlers, A. Zieske, G. Plenz, H. Robenek, P. Salbach, H. Kuhn, O. Radmark, B. Samuelsson, A.J. Habenicht, Expanding expression of the 5-lipoxygenase pathway within the arterial wall during human atherogenesis, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 1238–1243.
[19] J. Roos, C. Oancea, M. Heinssmann, D. Khan, H. Held, A.S. Kahnt, R. Capelo, E. la Buscato, E. Proschak, E. Puccetti, D. Steinhilber, I. Fleming, T.J. Maier, M. Ruthardt, 5-lipoxygenase is a candidate target for therapeutic management of stem cell-like cells in acute myeloid leukemia, Cancer Res. 74 (2014) 5244–5255.
[20] M. Back, Leukotriene signaling in atherosclerosis and ischemia, Cardiovasc. Drugs Ther. 23 (2009) 41–48.
[21] M. Back, A. Sultan, O. Ovchinnikova, G.K. Hansson, 5-Lipoxygenase-activating protein: a potential link between innate and adaptive immunity in atherosclerosis and adipose tissue inflammation, Circ. Res. 100 (2007) 946–949.
[22] J. Jawien, M. Gajda, M. Rudling, L. Mateuszuk, R. Olszanecki, T.J. Guzik, T. Cichocki, S. Chlopicki, R. Korbut, Inhibition of five lipoxygenase activating protein (FLAP) by MK-886 decreases atherosclerosis in apoE/LDLR-double knockout mice, Eur. J. Clin. Investig. 36 (2006) 141–146.
[23] H. Qiu, A. Gabrielsen, H.E. Agardh, M. Wan, A. Wetterholm, C.H. Wong, U. Hedin, J. Swedenborg, G.K. Hansson, B. Samuelsson, G. Paulsson-Berne, J.Z. Haeggstrom, Expression of 5-lipoxygenase and leukotriene A4 hydrolase in human atherosclerotic lesions correlates with symptoms of plaque instability, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 8161–8166.
[24] S. Sarveswaran, C.E. Myers, J. Ghosh, MK591, a leukotriene biosynthesis inhibitor, induces apoptosis in prostate cancer cells: synergistic action with LY294002, an inhibitor of phosphatidylinositol 3′-kinase, Cancer Lett. 291 (2010) 167–176.
[25] S. Sarveswaran, V. Thamilselvan, C. Brodie, J. Ghosh, Inhibition of 5-lipoxygenase triggers apoptosis in prostate cancer cells via down-regulation of protein kinase C-epsilon, Biochim. Biophys. Acta 1813 (2011) 2108–2117.
[26] X. Chen, S. Sood, C.S. Yang, N. Li, Z. Sun, Five-lipoxygenase pathway of arachidonic acid metabolism in carcino-genesis and cancer chemoprevention, Curr. Cancer Drug Targets 6 (2006) 613–622.
[27] Y. Chen, D. Li, S. Li, The Alox5 gene is a novel therapeutic target in cancer stem cells of chronic myeloid leukemia, Cell Cycle 8 (2009) 3488–3492.
[28] T.J. Kennedy, C.Y. Chan, X.Z. Ding, T.E. Adrian, Lipoxygenase inhibitors for the treatment of pancreatic cancer, Expert Rev. Anticancer Ther. 3 (2003) 525–536.
[29] E. Banoglu, B. Caliskan, S. Luderer, G. Eren, Y. Ozkan, W. Altenhofen, C. Weinigel, D. Barz, J. Gerstmeier, C. Pergola, O. Werz, Identification of novel benzimidazole derivatives as inhibitors of leukotriene biosynthesis by virtual screening targeting 5-lipoxygenase-activating protein (FLAP), Bioorg. Med. Chem. 20 (2012) 3728–3741.
[30] B. Caliskan, S. Luderer, Y. Ozkan, O. Werz, E. Banoglu, Pyrazol-3-propanoic acid derivatives as novel inhibitors of leukotriene biosynthesis in human neutrophils, Eur. J. Med. Chem. 46 (2011) 5021–5033.
[31] R. Sardella, S. Levent, F. Ianni, B. Caliskan, J. Gerstmeier, C. Pergola, O. Werz, E. Banoglu, B. Natalini, Chromatographic separation and biological evaluation of benzimidazole derivative enantiomers as inhibitors of leukotriene biosynthesis, J. Pharm. Biomed. Anal. 89 (2014) 88–92.
[32] C. Pergola, J. Gerstmeier, B. Monch, B. Caliskan, S. Luderer, C. Weinigel, D. Barz, J. Maczewsky, S. Pace, A. Rossi, L. Sautebin, E. Banoglu, O. Werz, The novel benzimidazole derivative BRP-7 inhibits leukotriene biosynthesis in vitro and in vivo by targeting 5-lipoxygenase-activating protein (FLAP), Br. J. Pharmacol. 171 (2014) 3051–3064.
[33] G. Eren, A. Macchiarulo, E. Banoglu, From molecular docking to 3D-quantitative structure-activity relationships (3D-QSAR): insights into the binding mode of 5-lipoxygenase inhibitors, Mol. Inf. 31 (2012) 123–134.
[34] M.G. Chini, R. De Simone, I. Bruno, R. Riccio, F. Dehm, C. Weinigel, D. Barz, O. Werz, G. Bifulco, Design and synthesis of a second series of triazole-based compounds as potent dual mPGES-1 and 5-lipoxygenase inhibitors, Eur. J. Med. Chem. 54 (2012) 311–323.
[35] N.C. Gilbert, S.G. Bartlett, M.T. Waight, D.B. Neau, W.E. Boeglin, A.R. Brash, M.E. Newcomer, The structure of human 5-lipoxygenase,, Science 331 (2011) 217–219.
[36] A.G. Habeeb, P.N. Praveen Rao, E.E. Knaus, Design and synthesis of 4,5-diphenyl-4-isoxazolines: novel inhibitors of cyclooxygenase-2 with analgesic and antiinflammatory activity, J. Med. Chem. 44 (2001) 2921–2927.
[37] P.A. Brough, W. Aherne, X. Barril, J. Borgognoni, K. Boxall, J.E. Cansfield, K.M. Cheung, I. Collins, N.G. Davies, M.J. Drysdale, B. Dymock, S.A. Eccles, H. Finch, A. Fink, A. Hayes, R. Howes, R.E. Hubbard, K. James, A.M. Jordan, A. Lockie, V. Martins, A. Massey, T.P. Matthews, E. McDonald, C.J. Northfield, L.H. Pearl, C. Prodromou, S. Ray, F.I. Raynaud, S.D. Roughley, S.Y. Sharp, A. Surgenor, D.L. Walmsley, P. Webb, M. Wood, P. Workman, L. Wright, 4,5-diarylisoxazole Hsp90 chaperone inhibitors: potential therapeutic agents for the treatment of cancer, J. Med. Chem. 51 (2008) 196–218.
[38] O. Werz, D. Steinhilber, Therapeutic options for 5-lipoxygenase inhibitors, Pharmacol. Ther. 112 (2006) 701–718.
[39] A.D. Ferguson, B.M. McKeever, S. Xu, D. Wisniewski, D.K. Miller, T.T. Yamin, R.H. Spencer, L. Chu, F. Ujjainwalla, B.R. Cunningham, J.F. Evans, J.W. Becker, Crystal structure of inhibitor-bound human 5-lipoxygenase-activating protein, Science 317 (2007) 510–512.
[40] R. De Simone, M.G. Chini, I. Bruno, R. Riccio, D. Mueller, O. Werz, G. Bifulco, Structure-based discovery of inhibitors of microsomal prostaglandin E2 synthase-1, 5-lipoxygenase and 5-lipoxygenase-activating protein: promising hits for the development of new anti-inflammatory agents, J. Med. Chem. 54 (2011) 1565–1575.
[41] A.D. Ferguson, Structure-based drug design on membrane protein targets: human integral membrane protein 5-lipoxygenase-activating protein, Methods Mol. Biol. 841 (2012) 267–290.
[42] J.F. Evans, A.D. Ferguson, R.T. Mosley, J.H. Hutchinson, What's all the FLAP about?: 5-lipoxygenase-activating protein inhibitors for inflammatory diseases, Trends Pharmacol. Sci. 29 (2008) 72–78.
[43] M.A. Lomize, A.L. Lomize, I.D. Pogozheva, H.I. Mosberg, OPM: orientations of proteins in membranes database, Bioinformatics 22 (2006) 623–625.
[44] C. Kandt, W.L. Ash, D.P. Tieleman, Setting up and running molecular dynamics simulations of membrane proteins, Methods 41 (2007) 475–488.
[45] A.M. Schaible, H. Traber, V. Temml, S.M. Noha, R. Filosa, A. Peduto, C. Weinigel, D. Barz, D. Schuster, O. Werz, Potent inhibition of human 5-lipoxygenase and microsomal prostaglandin E synthase-1 by the anti-carcinogenic and anti-inflammatory agent embelin, Biochem. Pharmacol. 30 (1) (2016) 276–285.
[46] L. Fischer, D. Szellas, O. Radmark, D. Steinhilber, O. Werz, Phosphorylation- and stimulus-dependent inhibition of cellular 5-lipoxygenase activity by nonredox-type inhibitors, FASEB J. 17 (2003) 949–951.
[47] O. Werz, E. Burkert, B. Samuelsson, O. Radmark, D. Steinhilber, Activation of 5-lipoxygenase by cell stress is calcium independent in human polymorphonuclear leukocytes, Blood 99 (2002) 1044–1052.