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Brief Article

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Synthesis and Structure−Activity Relationship Studies of Novel Dual Inhibitors of Soluble Epoxide Hydrolase and 5-Lipoxygenase

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Supporting Information

ABSTRACT: Current research leads to the assumption that drugs affecting more than one target could result in a more efficient treatment of diseases and fewer safety concerns. Administration of drugs inhibiting only one branch of the arachidonic acid cascade is usually accompanied by side effects. We therefore designed and synthesized a library of hybrid molecules incorporating an imidazo[1,2-*a*]pyridine and an urea moiety as novel soluble epoxide hydrolase (sEH)/5-lipoxygenase (5-LO) dual inhibitors. Evaluation of the compounds was accomplished by in vitro testing using recombinant enzyme assays.

INTRODUCTION

The approach "one drug−one target−one disease" was the predominant principle in drug development over decades. However, when considering treatment of complex diseases, interaction with one target may not be enough. Clinical studies on approved drugs showed that we may consider a rational design of drugs that interact with multiple targets but do not interact with off-targets responsible for side effects. These so-called designed multiple ligands (DMLs) have the advantage over combination therapies that there are fewer safety concerns because drug−drug interactions need not be considered.[1]

The arachidonic acid cascade is involved in many inflammatory processes due to the production of diverse inflammatory mediators such as prostaglandins and leukotrienes, products of the cyclooxygenase (COX) and the lipoxygenase (LOX) pathways.[2] The 5-lipoxygenase (5-LO) is a dioxygenase that catalyzes two steps in the biosynthesis of leukotrienes which regulate the innate immune response and play a pathophysiological role in chronic inflammatory diseases such as asthma and atherosclerosis.[3] As reviewed by Pergola and Werz,[4] 5-LO inhibitors have been developed for over 25 years, but the only inhibitor that entered the US market yet, is Zileuton (Figure 1), a drug used for the treatment of asthma with restrictions due to its liver toxicity and a short half-life.[5] Recent research therefore focuses on the potential of dual inhibitors such as those for prostaglandin/leukotriene synthesis. An example for this is licofelone[6] (Figure 1), which inhibits the 5-LO activating protein (FLAP), the COX-1, and the microsomal prostaglandin E synthase-1 (mPGES-1).[4,7]

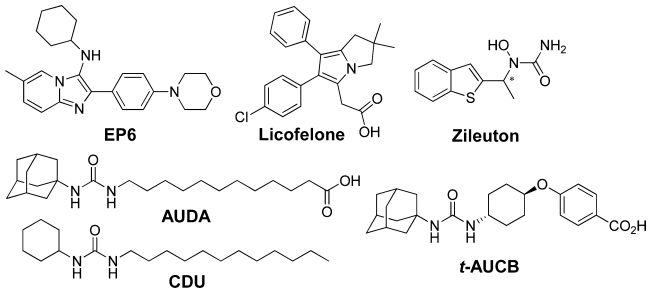

Figure 1. Known inhibitors of sEH and 5-LO.

Besides the COX and LOX pathways, CYP enzymes also metabolize arachidonic acid to either hydroxyeicosatetraenoic acids (HETEs) or epoxyeicosatrienoic acids (EETs). The latter are further hydrolyzed by the soluble epoxide hydrolase (sEH) to their corresponding diols, the dihydroxyeicosatrienoic acids (DHETs).[8−10] EETs exhibit anti-inflammatory and antihypertensive properties and have been reported to exert beneficial effects in disease models related to cardiovascular diseases, diabetes, inflammatory pain, and other indications.[8,10]

Both sEH$^{-/-}$ mice and animals treated with sEH inhibitors show an increased level of EETs and 5-LO products, indicating that sEH inhibitors might synergize with COX- and 5-LO inhibitors. Co-administration of the sEH inhibitor *t*-AUCB (Figure 1) with either a COX- or 5-LO inhibitor showed a significant enhancement of their anti-inflammatory activities.[11] sEH inhibitors alone induced albuminuria in mice, probably due to a shift toward the 5-LO branch of the arachidonic acid cascade.[12] Hwang et al. developed dual COX-2/sEH inhibitors and showed in a lipopolysaccharide induced mouse model that the antiallodynic activity of the dual inhibitor is more effective than either single-target inhibitor alone or the coadministration of a COX-2 and a sEH inhibitor.[13]

Therefore, the development of DMLs inhibiting both 5-LO and sEH might lead not only to highly interesting and effective anti-inflammatory compounds, but also to safer antihypertensive drugs. We synthesized hybrid molecules out of two known pharmacophores of sEH and 5-LO inhibitors to generate a tool compound for the further evaluation of this hypothesis.

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RESULTS AND DISCUSSION

Chemistry. The recently published imidazo[1,2-*a*]pyridine EP6 (Figure 1) was used as backbone for the 5-LO pharmacophore, with variations on four moieties (Figure 2).[14] Urea compounds are a well-known class of sEH inhibitors.[10] Besides adamantyl (F) and cyclohexyl (E) residues, known from AUDA[10] and CDU[10] (Figure 1), we also introduced *para*-substituted phenyl groups (A–D) that showed improved pharmacokinetic properties in animal studies.[15] The linkage between these two pharmacophores was achieved with either a piperidine ring or a propyl linker (Figure 2).[10,13,16]

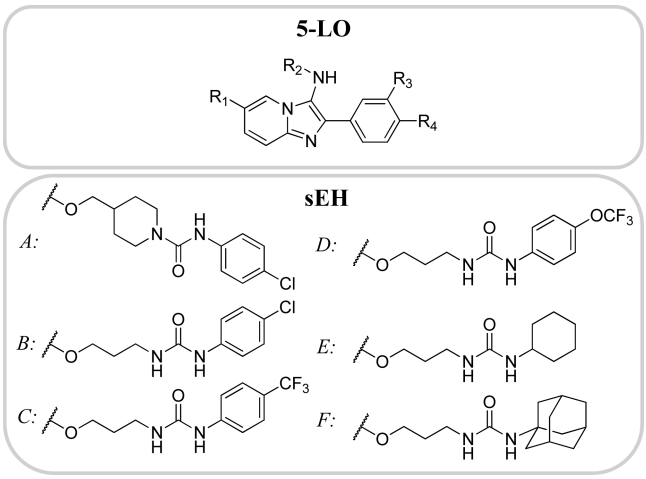

Figure 2. Combinatorial strategy for dual inhibitors of sEH/5-LO. R_1 = −Me, −F, −Cl; R_2 = −^{i}propyl, −cyclopentyl, −cyclohexyl; R_3 = −H, −OMe, −Cl, *C*, *D*; R_4 = −OMe, *A*, *B*, *C*, *D*, *E*, *F*.

In both cases, formation of the urea (**2**, **6a–e**) was achieved by reacting an amine with an isocyanate.[17] The secondary urea incorporating the piperidine ring **2** underwent a Mitsunobu reaction[18] with 4-hydroxybenzaldehyde to form the desired intermediate **3**. The last step was a Groebke–Blackburn–Bienaymé multicomponent reaction[19] with an isonitrile and 6-amino-3-picoline under catalytic acidic conditions to form the desired DMLs **4a–b** (Table 1). In contrast to the Mitsunobu reaction, ureas **6a–e** reacted with a hydroxybenzaldehyde derivative in a Williamson ether synthesis[20] to form intermediates **7a–g**. Formation of DMLs **8a–t** was accomplished in the same way as for compounds **4a–b** with the difference of using diverse amino pyridine derivatives (Scheme 1).

Variation of the sEH Backbone Introducing a Different Linker. We therefore implemented a propyl linker between the two pharmacophores, combined with variations on the terminal hydrophobic moiety, as better inhibitory potential and better pharmacokinetics have been postulated for *para*-substituted aryl groups with either a trifluoromethyl (C) or a trifluoromethoxy (D) group.[13,15] The new linker increased

Table 1. Inhibitory Evaluation of Compounds 4a–b and 8a–t in Vitro with Recombinant Human Enzymes

					IC_{50}[a] [nM] ± SD	
entry	R_1	R_2	R_3	R_4	5-LO	sEH
EP6					59 ± 22.9	>10000
Zileuton					591 ± 63.5	
AUDA						107 ± 14.7
2					>10000	2237 ± 495.4
4a	Me	cyclohexyl	H	*A*	106 ± 24.9	1467 ± 153.7
4b	Me	isopropyl	H	*A*	65 ± 15.9	1540 ± 155.8
8a	Me	cyclohexyl	OMe	*B*	70 ± 16.3	842 ± 20.0
8b	Me	cyclohexyl	OMe	*C*	44 ± 7.0	439 ± 97.3
8c	Me	cyclohexyl	OMe	*D*	63 ± 15.8	116 ± 14.7
8d	Cl	cyclohexyl	OMe	*D*	32 ± 4.2	191 ± 26.7
8e	Cl	cyclohexyl	OMe	*C*	30 ± 7.6	336 ± 26.1
8f	F	cyclohexyl	OMe	*D*	82 ± 20.1	696 ± 112.7
8g	F	cyclohexyl	OMe	*C*	52 ± 10.1	649 ± 140.4
8h	Me	cyclopentyl	OMe	*D*	71 ± 11.1	473 ± 47.2
8i	Me	cyclopentyl	OMe	*C*	70 ± 18.9	614 ± 51.9
8j	Me	isopropyl	OMe	*D*	147 ± 15.2	558 ± 91.1
8k	Me	isopropyl	OMe	*C*	201 ± 73.5	620 ± 54.7
8l	Cl	cyclopentyl	OMe	*C*	92 ± 47.6	459 ± 91.8
8m	F	cyclopentyl	OMe	*C*	42 ± 6.5	480 ± 156.5
8n	Cl	isopropyl	OMe	*C*	104 ± 83.8	439 ± 52.1
8o	Me	cyclohexyl	*D*	OMe	38 ± 8.7	1107 ± 102.1
8p	Me	cyclohexyl	*C*	OMe	38 ± 16.5	1593 ± 177.5
8q	Me	cyclohexyl	H	*C*	62 ± 8.5	253 ± 57.0
8r	Me	cyclohexyl	Cl	*C*	189 ± 116.7	192 ± 32.7
8s	Me	cyclohexyl	OMe	*F*	100 ± 34.5	189 ± 60.6
8t	Me	cyclohexyl	OMe	*E*	242 ± 81.3	190 ± 41.6

[a] IC_{50} values are means with SD from at least three independent experiments.

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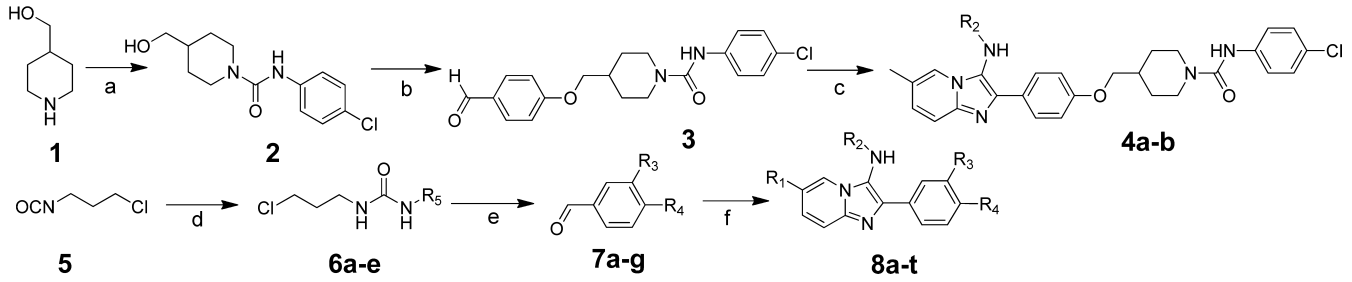
Scheme 1. Synthesis of Compounds 4a−b and 8a−t[a]



[a](a) Chlorophenylisocyanate, DCM, 0 °C to RT, 20 min, quant; (b) 4-hydroxybenzaldehyde, TPP, DIAD, THF, argon, 0 °C to RT, overnight, 60%; (c) R_2-isonitrile, 6-amino-3-picoline, AcOH, MeOH, RT, overnight, 19−22%; (d) R-amine, DCM, 0 °C to RT, overnight, 64−99%; (e) 3-R_3-4-hydroxybenzaldehyde or 3-hydroxy-4-methoxybenzaldehyde, Cs_2CO_3, DMF, 70 °C, overnight, 8−56%; (f) R_2-isonitrile, 2-amino-5-R_1-pyridine, AcOH, MeOH, overnight, 7−64%. R = *p*-Cl-phenyl, *p*-CF_3-phenyl, *p*-OCF_3-phenyl, −adamantyl, −cyclohexyl; R_1 = −Me, −F, −Cl; R_2 = −^{i}propyl, −cyclopentyl, −cyclohexyl; R_3 = −H, −OMe, −Cl, *C*, *D*; R_4 = −OMe, *A*, *B*, *C*, *D*, *E*, *F*.

sEH inhibition 2-fold (**8a**, IC_{50} = 842 nM) compared to **4a** and **4b**, whereas inhibition of 5-LO was maintained. Introduction of the trifluoromethyl group (**8b**) raised the sEH inhibition again 2-fold, while incorporation of the trifluoromethoxy group into the molecule (**8c**) gave a further advance to sEH inhibition, leading to the best IC_{50} value of 116 nM. 5-LO inhibition remained constant throughout these modifications (**8b**, IC_{50} = 44 nM; **8c**, IC_{50} = 63 nM). Therefore, a propyl spacer is favorable for the continuing design of inhibitors, in analogy to the studies by Hwang et al.[13]

SAR of Dual Inhibitors Incorporating a Propyl Linker. From this starting point (**8a−8c**), we developed further variations of inhibitors, modifying each moiety in order to enhance inhibitory potential. The methyl group in the position R_1 was exchanged by fluorine or chlorine. While sEH inhibition was mostly unaffected by chlorine (**8d**, IC_{50} = 191 nM; **8e**, IC_{50} = 336 nM), introduction of fluorine (**8f**, IC_{50} = 696 nM; **8g**, IC_{50} = 649 nM) diminished the inhibitory potential of the compounds. Also, for 5-LO inhibition, introduction of chlorine (**8d**, IC_{50} = 32 nM; **8e**, IC_{50} = 30 nM) was preferred over fluorine (**8f**, IC_{50} = 82 nM; **8g**, IC_{50} = 52 nM).

The next step was the implementation of smaller aliphatic residues in moiety R_2. We observed the tendency for sEH that cyclohexyl (**8b**, **8c**) is preferred over cyclopentyl (**8h**, **8i**) or isopropyl (**8j**, **8k**), indicating that a larger moiety improves the inhibitory potential. Cyclohexyl and cyclopentyl, with comparable values, were also preferred on 5-LO over an isopropyl residue. Combination of fluorine or chlorine in moiety R_1 with cyclopentyl (**8l**, IC_{50} = 459 nM, **8m**, IC_{50} = 480 nM) or isopropyl (**8n**, IC_{50} = 439 nM) in moiety R_2 led to a decrease in sEH inhibition relative to the best inhibitor **8c**. But when comparing **8l**, **8m**, and **8n** to compounds with a single modification on either R_1 or R_2 (**8d**, **8g**, **8i**, **8k**), sEH inhibition was maintained or increased. For 5-LO, combination of fluorine with cyclopentyl **8m** is comparable to other potent inhibitors (**8b**, **8d**, **8e**, **8g**), showing an IC_{50} value of 42 nM.

A connection between the two pharmacophores in the *meta* position (**8o**, **8p**) led to equivalent values in terms of 5-LO inhibition, but was not tolerated by sEH, possibly due to sterical hindrance. Besides *meta* substitution, modifications on moiety R_3 showed that either a methoxy group (**8b**, IC_{50} = 44 nM) or a hydrogen atom (**8q**, IC_{50} = 62 nM) was preferred over a chlorine (**8r**, IC_{50} = 189 nM) for 5-LO, whereas for sEH, the chlorine (**8r**, IC_{50} = 192 nM) and the hydrogen atom (**8q**, IC_{50} = 253 nM) were favored over the methoxy group (**8b**, IC_{50} = 439 nM).

Modifications of R_4 with Nonaromatic Residues (E,F). Compounds such as AUDA and CDU showed that an aromatic moiety is not necessary for the inhibition of sEH.[10] We therefore combined our 5-LO backbone with an urea connected with a propyl linker to either an adamantyl (F, **8s**) or a cyclohexyl (E, **8t**) moiety. While IC_{50} values for sEH inhibition were constant at 189 and 190 nM, slightly lower than the best compound **8c**, we obtained a decrease in 5-LO inhibition (**8s**, IC_{50} = 100 nM; **8t**, IC_{50} = 242 nM) compared to **8a−c**.

Taken together, we developed dual inhibitors with adequate inhibitory potential toward both target enzymes. Optimization on the target 5-LO led to IC_{50} values in a nanomolar range, 30 nM being the best value (**8e**). If we compare the dual inhibitor **8e** with the 5-LO inhibitor EP6 (59 nM), the values are in the same range, showing that the sEH inhibitor part of **8e** is tolerated by the 5-LO very well. Inhibition of the sEH reached values in the range of 116−192 nM (**8c**, **8d**, **8r**, **8s**, **8t**), which are also comparable to AUDA (107 nM). Compounds **8r** and **8t** are equipotent on the two targets, whereas **8c** and **8d** combine the best sEH inhibitory potency with one of the best potentials toward 5-LO.

CONCLUSION

We report the rational design and synthesis of dual inhibitors of sEH and 5-LO. Compounds synthesized for a SAR showed good inhibitory potential on recombinant enzyme for both targets. In analogy to previous studies,[13,21] it was possible to incorporate the sEH pharmacophore into a dual-target inhibitor. These compounds can be used as tools to further assess the idea of adopting dual sEH/5-LO inhibitors as drugs targeting several disease models. Whether an equipotent inhibition (**8r**, **8t**) is more favorable over a higher inhibition on one of the two targets (**8c**, **8d**) needs to be evaluated.

EXPERIMENTAL SECTION

General. All reagents and solvents were purchased from the suppliers Sigma-Aldrich Chemistry GmbH (Hannover, Germany) or Alfa-Aesar GmbH & Co. KG (Karlsruhe, Germany) and were used without further purification. Flash chromatography was performed on packed silica columns (particle size 50 μm) from Varian Medical Systems GmbH (Darmstadt, Germany). NMR spectra were measured on AV 250 and AV 300 nuclear magnetic resonance spectrometers from Bruker. Chemical shifts are reported in parts per million (ppm) using TMS as internal standard: ^{1}H (250/300 MHz), ^{13}C (63/75 MHz), ^{19}F (282 MHz). Mass spectra were measured using ESI with a VG Platform II spectrometer by Fisons or a Mariner Biospectrometry

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workstation by Perspective Biosystems. HRMS spectra were measured by a MALDI LTQ Orbitrap XL spectrometer from Thermo Scientific. Combustion analysis was performed on an Elementar Vario Micro Tube CHNO rapid elemental analyzer. HPLC was performed on a LC2020 (Shimadzu, Duisburg, Germany), conditions: water/methanol gradient run 5−95% using a Kinetex 2.6 μm C18 100 Å, 100 mm × 2.1 mm (Phenomenex, Aschaffenburg, Germany) at room temperature, UV detection at 254 and 280 nm. All compounds were characterized by ^{1}H NMR and MS, final compounds additionally by ^{13}C NMR, ^{19}F NMR, and HRMS, and combustion analysis or HPLC to determine purity >95%. Activity assays for sEH[22] and 5-LO[23] were performed according to earlier publications and are detailed in the Supporting Information. All IC_{50} values are means with SD obtained from measurements at six−eight different concentrations of the compounds from at least three separate experiments. IC_{50} values were determined using a four-parameter sigmoidal dose response equation (in the case of Zileuton, five different concentrations from three separate experiments were used to fit a three-parameter sigmoidal dose response equation) from GraphPad Prism software (GraphPad Software, LaJolla, CA, USA) and constitute relative IC_{50} values.

*General Procedure d for Compounds **6a−e**.* 3-Chloropropylisocyanate (**5**) (1 equiv) was added to a solution of trifluoromethylaniline (or other aniline derivative, 1 equiv) in DCM at 0 °C. The mixture was allowed to warm to room temperature and was stirred overnight. A yellow solution was obtained. The solvent was removed under reduced pressure and the white solid was triturated with hexane to form the pure product.

*General Procedure e for Compounds **7a−i**.* Vanillin (or derivative, 1 equiv) and cesium carbonate (1 equiv) in 40 mL of DMF were heated to 70 °C for 30 min. Compound **6a−e** (1 equiv), dissolved in DMF, was added, and the mixture was stirred overnight at 70 °C. The solvent was removed, and the residue was diluted in 20 mL of EtOAc. The organic phase was extracted with 20 mL of water, twice with 20 mL of NaOH solution (1M), and again with 20 mL of water. After drying the organic layer over $MgSO_4$, the solvent was removed under reduced pressure. The product was recrystallized from hexane/EtOAc. If necessary, a further recrystallization from EtOAC/EtOH or flash column chromatography with the same solvents was performed in order to obtain the pure products.

*General Procedure c for Compound **4b** and f for Compounds **8a−t**.* 6-Amino-3-picoline (or other pyridine amine, 1.2 equiv) and **3** or **7a−i** (1 equiv) were stirred with glacial acetic acid in 20 mL of MeOH, and then *N*-cyclohexylisonitrile (or other isonitrile, 1 equiv) was added at 0 °C. After stirring overnight at room temperature, the reaction mixture was quenched with 2N HCl. The solvent was removed under reduced pressure. The residue was solved in 20 mL of EtOAc and washed (3 × 20 mL) with saturated $NaHCO_3$. The organic phase was dried over $MgSO_4$, the solvent removed under reduced pressure, and the crude product was recrystallized from hexane/EtOAc or by flash column chromatography using hexane/EtOAc or EtOAc/EtOH.

*Synthesis of N-(4-Chlorophenyl)-4-(hydroxymethyl)piperidine-1-carboxamide (**2**).* 4-Chlorophenylisocyanate (0.33 g, 2.15 mmol) was slowly added to a solution of 4-piperidinemethanol (**1**) (0.25 g, 2.17 mmol) in DCM at 0 °C. The mixture was allowed to warm to room temperature and was stirred for further 20 min. The solvent was removed and the crude product was triturated with hexane to form the pure product as a white solid (0.55 g, 2.03 mmol, 94%).

*Synthesis of N-(4-Chlorophenyl)-4-((4-formylphenoxy)methyl)-piperidine-1-carboxamide (**3**).* **2** (0.25 g, 0.93 mmol), 4-hydroxybenzaldehyde (0.1 g, 0.78 mmol), and TPP (0.25 g, 0.97 mmol) were stirred in 20 mL of anhydrous DMF under argon atmosphere in an ice bath. DIAD (0.19 mL, 0.97 mmol) was added dropwise in 10 mL of DMF. After 24 h, the solvent was removed under reduced pressure and the crude mixture was purified by flash column chromatography using hexane:EtOAc 2:3.The product was isolated as an off-white solid (0.17g, 0.47 mmol, 60.2%).

*Synthesis of N-(4-Chlorophenyl)-4-((4-(3-(cyclohexyl-amino)-6-methylimidazo[1,2-a]pyridin-2-yl)phenoxy)-methyl)piperidine-1-carboxamide (**4a**).* 6-Amino-3-picoline (0.03 g, 0.25 mmol) and **4** (0.08 g, 0.21 mmol) were stirred with glacial acetic acid (0.01 mL, 0.21 mmol) in 20 mL of MeOH, and then *N*-cyclohexylisonitrile (0.02 g, 0.21 mmol) was added. The mixture was stirred overnight at room temperature. The reaction was quenched with 2N HCl. The solvent was removed under reduced pressure. The residue was solved in 20 mL of EtOAc and washed (3 × 20 mL) with saturated $NaHCO_3$. The organic phase was dried over $MgSO_4$, the solvent was removed under reduced pressure, and the crude product was recrystallized from EtOH. The product was isolated as a yellow solid (26 mg, 0.05 mmol, 22%). ^{1}H NMR (250 MHz, DMSO-d_6): δ 8.6 (s, 1H), 8.15−8.06 (m, 2H), 7.51 (d, J = 16.7 Hz, 2H), 7.35−7.27 (m, 3H), 7.01−6.96 (m, 3H), 4.15 (d, J = 21.5 Hz, 2H), 3.89 (d, J = 6.4 Hz, 2H), 2.9−2.78 (m, 1H), 2.31 (s, 3H), 2.02−1.92 (m, 1H), 1.84−1.48 (m, 9H), 1.27−1.02 (m, 9H). ^{13}C NMR (75 MHz, DMSO-d_6): 157.6, 154.6, 139.7, 139.5, 134.9, 128.0, 127.6, 127.4, 126.4, 125.1, 124.3, 120.8, 120.5, 120.1, 115.9, 114.1, 71.7, 56.2, 43.6, 35.6, 33.4, 28.5, 25.4, 24.5, 17.8. MS (ESI, 70 eV) m/z (%): 573.0 (100) (M + H). Anal. Calcd for $C_{33}H_{38}ClN_5O_2 \cdot 0.25HCl$: C, 68.19, H, 6.63, N, 12.05. Found: C, 68.44, H, 6.54, N, 12.06.

Synthesis and analytical data for compounds **4a−b** and **8a−t** and their intermediates are detailed in the Supporting Information.

sEH Activity Assay. For the determination of inhibitory potential of our compounds, we used recombinant, affinity-purified human sEH from *Escherichia coli*. The assay setup was as previously described,[22] using a fluorescence-based assay with the substrate 3-phenyl-cyano(6-methoxy-2-naphthalenyl)methylester-2-oxiraneacetic acid (PHOME). IC_{50} values were determined in a 96-well format utilizing 2 μg/well recombinant sEH. After incubation with inhibitors for 30 min at room temperature in 25 mM Bis-Tris/HCl, 0.1 mg/mL BSA, and 0.01% TritonX 100 buffer (110 μL, pH 7.0), the substrate (PHOME) was added ($[S]_{final}$ = 50 μM). Activity was evaluated by measuring the appearance of the fluorescent product 6-methoxy-2-naphthaldehyde (λ_{em} = 330 nm, λ_{ex} = 465 nm).

5-LO Activity Assay. The evaluation of the activity on purified 5-LO was accomplished with the method published by Brungs et al.[23] Therefore, 2−3 μg of partially purified human 5-LO from *E. coli* were diluted in 1 mL of 1 mM PBS/EDTA solution. Preincubation with the test compounds or vehicle (DMSO) for 15 min at 4 °C was followed by prewarming for 30 s at 37 °C. Afterward, the reaction was started by the addition of 2 mM $CaCl_2$ and 20 μM AA at 37 °C. Ice-cold MeOH (1 mL) was added to stop the reaction after 10 min. Metabolites formed during the reaction were analyzed by HPLC.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthesis and analytical data of all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

t-AUCB, *trans*-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyl-oxy]-benzoic acid; AUDA, 12-(3-adamantan-1-yl-ureido)dodecanoic

acid; CDU, 1-cyclohexyl-3-dodecyl urea; DHET, dihydroxyeicosatrienoic acid; DML, designed multiple ligand; EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetranoic acid; 5-LO, 5-lipoxygenase; 5-LO-p, 5-LO pharmacophore; FLAP, 5-LO activating protein; mPGES-1, microsomal prostaglandin E synthase-1; sEH, soluble epoxide hydrolase

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