Identification of Natural-Product-Derived Inhibitors of 5-Lipoxygenase Activity by Ligand-Based Virtual Screening

Lutz Franke,[†] Oliver Schwarz,[‡] Lutz Müller-Kuhrt,[‡] Christina Hoernig,[§] Lutz Fischer,[§] Sven George,[§] Yusuf Tanrikulu,[†] Petra Schneider,[||] Oliver Werz,[⊥] Dieter Steinhilber,[§] and Gisbert Schneider*[,†]

Institut für Organische Chemie und Chemische Biologie/ZAFES, Johann Wolfgang Goethe-Universität, Siesmayerstrasse 70, D-60323 Frankfurt am Main, Germany, AnalytiCon Discovery GmbH, Hermannswerder Haus 17, D-14473 Potsdam, Germany, Institut für Pharmazeutische Chemie/ZAFES, Johann Wolfgang Goethe-Universität, Max-von-Laue Strasse 9, D-60438 Frankfurt am Main, Germany, Schneider Consulting GbR, George-C.-Marshall Ring 33, D-61440 Oberursel, Germany, and Pharmazeutisches Institut, Universität Tuebingen, Auf der Morgenstelle 8, D-72076 Tuebingen, Germany

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A natural product collection and natural-product-derived combinatorial libraries were virtually screened for potential inhibitors of human 5-lipoxygenase (5-LO) activity. We followed a sequential ligand-based approach in two steps. First, similarity searching with a topological pharmacophore descriptor (CATS 2D method) was performed to enable scaffold-hopping. Eighteen compounds were selected from a virtual hit list of 430 substances, which had mutual pharmacophore features with at least one of 43 known 5-LO inhibitors that served as query structures. Two new chemotypes exhibited significant activity in a cell-based 5-LO activity assay. The two most potent molecules served as seed structures for a second virtual screening round. This time, a focused natural-product-derived combinatorial library was analyzed by different ligand-based virtual screening methods. The best molecules from the final set of screening candidates potently suppressed 5-LO activity in intact cells and may represent a novel class of 5-LO inhibitors. The results demonstrate the potential of natural-product-derived screening libraries for hit and lead structure identification.

Introduction

5-Lipoxygenase (5-LO)[a] catalyzes the first steps in the conversion of arachidonic acid into leukotrienes that are mediators of inflammatory and allergic reactions. Recently, the 5-LO pathway has also been associated with atherosclerosis, cancer, and osteoporosis,[1−3] so that 5-LO has become an attractive drug target. 5-LO inhibitors can be classified into three main groups: redox-active compounds, iron-ligand inhibitors with weak redox-active properties, and non-redox-type inhibitors. Early screening programs identified many redox-active compounds like nordihydroguaiaretic acid, coumarins, or flavonoids (e.g., cirsiliol) as 5-LO inhibitors. These compounds act as nonselective antioxidants that interrupt redox cycling of 5-LO due to radical scavenging properties and their ability to reduce the active-site ferric iron of the enzyme.[4]

The rational development of compounds that are able to chelate the active-site iron led to hydroxamic acid and *N*-hydroxyurea derivatives. The most prominent *N*-hydroxyurea derivative is A-64077 (Zileuton), which is the first and only 5-LO inhibitor up to now that is on the market for the treatment of asthma.[5] The disadvantage of redox-active inhibitors and iron ligands, that is, the participation in redox reactions and lack of selectivity, initiated the development of non-redox-type inhibitors.[6] It should be noted that the classification of compounds as non-redox-type inhibitors relates to the lack of redox activity of the drugs themselves but does not exclude that the inhibitory potency of the drugs is affected by the redox state of their target 5-LO. Most of the non-redox-type inhibitors may compete with arachidonic acid for binding to 5-LO and comprise structurally diverse molecules which are selective inhibitors of the 5-LO enzyme with respect to cyclooxygenases and 12- and 15-LOs.

The aim of our study was to search for potential novel 5-LO inhibitors in a natural product collection and natural-product-derived compound libraries. The selection of screening candidates was guided by ligand-based virtual screening due to lack of an experimentally obtained receptor structure. The study aimed at "scaffold-hopping": We compiled a diverse set of published 5-LO pathway inhibitors and used these as queries in a database scan for alternative chemotypes with 5-LO inhibitory activity. This ligand-based virtual screening approach consisted of two consecutive steps:

In a first round, natural products and natural-product-derived combinatorial compound collections were virtually screened with 43 queries that were taken from a collection of published bioactive compounds (COBRA collection, version 4.1).[7] The software speedCATS was applied for this purpose—a method that is grounded on a topological pharmacophore-pair descriptor.[8,9] For each of the 43 reference molecules, the 10 most similar molecules were retrieved from the AnalytiCon Discovery compound repository. From the resulting list of 430 candidates, 18 molecules were cherry-picked, ordered, and tested in a cellular and a cell-free assay system for inhibition of 5-LO product synthesis. The AnalytiCon compound collection was searched as it allowed us to pick pure natural

* Author to whom correspondence should be addressed: tel +49 (0) 69 798 24873/4; fax +49 (0) 69 798 24880; e-mail g.schneider@chemie.uni-frankfurt.de.

[†] Institut für Organische Chemie und Chemische Biologie/ZAFES, Johann Wolfgang Goethe-Universität.

[‡] AnalytiCon Discovery GmbH.

[§] Institut für Pharmazeutische Chemie/ZAFES, Johann Wolfgang Goethe-Universität.

[||] Schneider Consulting GbR.

[⊥] Universität Tuebingen.

[a] Abbreviations: 5-LO, 5-lipoxygenase; PMNL, polymorphonuclear leucocytes

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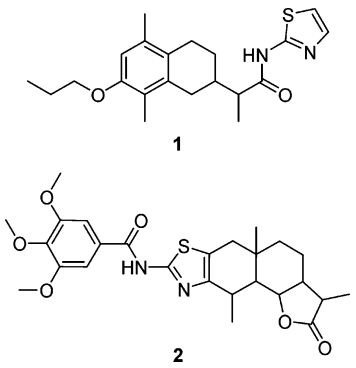
Chart 1. Most Potent Hits from the First Virtual Screening Round



products and natural-product-derived substances for screening, thereby avoiding the screening of compound mixtures or extracts.

In the second virtual screening round, the two most active molecules from round 1 served as queries for similarity searching in a focused combinatorial library. This was done to retrieve potentially more active substances and obtain a preliminary structure−activity relationship (SAR) model. This time, four different virtual screening methods were used (three variants of the CATS approach[9] and MACCS keys with the Tanimoto index),[10−12] and 17 compounds were subsequently tested for inhibition of 5-LO product synthesis.

As a result, two novel chemotypes were identified that exhibit nanomolar activity in intact polymorphonuclear leukocytes (PMNL).

Results and Discussion

Methodological Concept. The virtual screening study was grounded on the "similarity principle".[13] We collected known 5-LO inhibitors from the literature and performed similarity searching for analogues in two consecutive steps:

(1) In a first virtual screening round, the MEGx collection and NATx compound library of AnalytiCon Discovery (version 2004, www.ac-discovery.com) were virtually screened by use of 43 5-LO pathway inhibitors that were taken from the COBRA database (query structures are listed in Table S1 in the Supporting Information).[7] The software speedCATS was applied for this purpose.[9] The 10 most similar database molecules were retrieved for each of the 43 reference molecules. Eighteen molecules were cherry-picked from this list of 430 candidates: all compounds that occurred at least twice, plus molecules with the smallest distance to their respective query molecules (Table S2 in the Supporting Information). Initially, these substances were tested for inhibition of 5-LO product formation in intact PMNL, and for the most potent hits IC_{50} values were determined (Chart 1). It is noteworthy that the best hits **1** and **2** both have different scaffold architecture than their corresponding query molecules, indicating successful scaffold-hops from the respective queries (Chart 2).

(2) In the second virtual screening round, the two most active molecules from round 1 (**1** and **2**) were used as queries for similarity searching in AnalytiCon's NAT-5 compound collection, yielding 17 virtual hits (Chart 3). This was done to retrieve potentially more potent substances and to obtain a preliminary SAR model. This time, four different virtual screening methods were used (three variants of the CATS approach[9] and MACCS keys with Tanimoto index[10−12]).

Results of First Screening Round. In the first screening round, we found the two novel inhibitors **1** and **2** representing different structural classes. They exhibited submicromolar activity in intact PMNL [IC_{50} (**1**) = 0.8 μM, IC_{50} (**2**) = 0.9 μM], which had been stimulated with 2.5 μM ionophore A23187 and 20 μM arachidonic acid, as described under Materials and Methods. For crude 5-LO in cell-free assays or partially purified 5-LO enzyme, IC_{50} values were approximately 1 μM for compound **1** and >10 μM for compound **2**. The well-established iron ligand-type 5-LO inhibitor BWA4C[14] and the non-redox-type 5-LO inhibitor ZM230487[15] were used as controls to validate the assays and estimate the potency and molecular mode of action of the candidate compounds (Figure 1). Additional preliminary tests indicated that the potency of **1** and **2** is not influenced under reducing conditions, that is, in the presence of 1 mM dithiothreitol or 1 mM glutathione and 30 milliunits of gluthatione peroxidase in the assay.[16]

From these data we conclude that compound **1** is in fact an inhibitor of leukotriene biosynthesis in intact cells, which might be caused by direct inhibition of 5-LO. Compound **2** was shown to inhibit cellular leukotriene biosynthesis (IC_{50} = 0.9 μM), whereas in cell-free assays IC_{50} was above 10 μM. Stronger enzyme inhibition at higher concentrations (10 μM), and thus a reliable IC_{50} value, was not attained. Therefore, the mechanism of action for potent suppression of 5-LO in intact cells might not be primarily through direct inhibition of 5-LO enzymatic activity but instead could be due to additional interference with cellular components or mechanisms required for activation of 5-LO in the intact cell (discussed below).

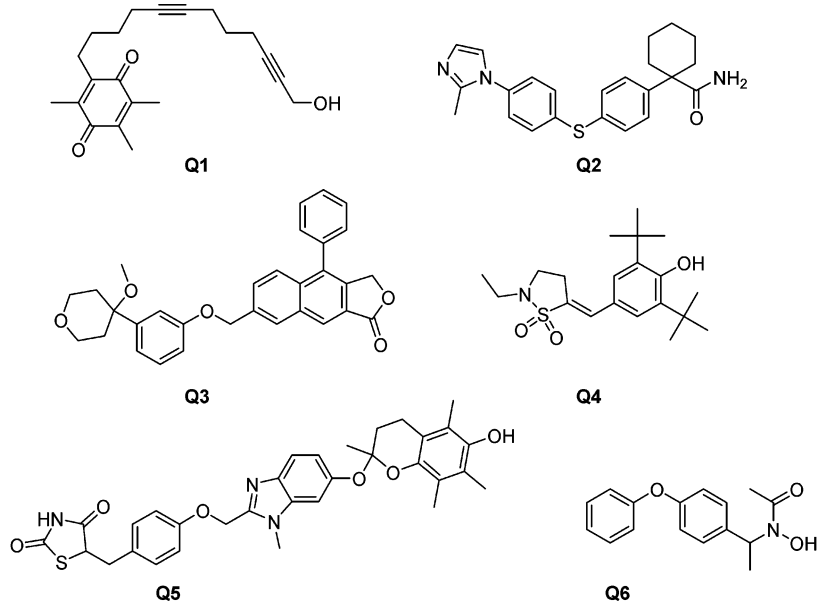
Results of Second Screening Round. Compounds **1** and **2** were retrieved from the AnalytiCon Discovery NAT-5 library. Therefore, virtual screening was restricted to NAT-5 compounds in the second screening cycle. This combinatorial library was built around scaffolds derived from α-santonin, a common sesquiterpenelactone occurring in different *Artemisia* species. Molecules **1** and **2** were used as reference molecules for similarity searching with the software tools speedCATS,[9] CATS-*Charge*,[9] CATS-*TripleCharge*, and MACCS keys.[11] Seventeen analogues (Chart 3) were retrieved and tested in intact PMNL, which were stimulated by 2.5 μM ionophore A23187 plus 20 μM arachidonic acid, and in the cell-free test system by use of partially purified 5-LO.

Molecules **3**−**10** are structural neighbors of compound **2**. Molecules **11**−**19** are analogues of **1**. In intact PMNL, none of the 17 analogues yielded higher activity than the queries at a concentration of 1 μM (Table 1). At 10 μM, compounds **8** and **9** had the strongest inhibitory effect with 14% and 19% remaining 5-LO activity, respectively. Overall, the inhibitory effect of molecules **3**−**10** was similar to that of compound **2**. IC_{50} values were below 10 μM in intact cells (Table 2).

As already observed for compound **2**, its analogues clearly suppressed partially purified 5-LO at 1 μM, without further inhibition at 10 μM. Compounds **9** and **10** both yielded IC_{50} values of approximately 1 μM, with remaining 5-LO activity of 55% and 46%, respectively. Why these two molecules exhibit this comparably strong effect in our cell-free assay cannot be immediately determined from their chemical structure. These results should thus be treated with care.

Analogues **11**−**19** of compound **1** exhibited lower inhibitory activity than the query in intact PMNL (Table 1). Only the incubation with molecule **11** resulted in an increased inhibition of 5-LO product formation (10% remaining 5-LO activity) at a concentration of 10 μM. At a concentration of 1 μM, the analogues of compound **1** were more potent in the cell-free 5-LO enzyme assay than in intact PMNL (Table 1). From these data we conclude that molecule **1** and its structural neighbors

Chart 2. Query Compounds That Led to Retrieval of New 5-LO Inhibitors **1**[a] and **2**[b]



[a] Found by queries Q1[29] and Q2.[30] [b] Found by queries Q3,[30] Q4,[31] Q5,[30] and Q6.[30]

might be regarded as direct 5-LO inhibitors, while no such statement can be made for molecule **2** and its derivatives. Although there is some direct inhibitory effect on the 5-LO enzyme, it seems likely that chemotype **2** affects 5-LO product formation by multiple mechanisms. Possible modes of action include inhibition of 5-LO-activating protein (FLAP),[17] interaction with the lipid-binding domain of 5-LO,[16] inhibition of 5-LO kinases,[18] or affecting 5-LO translocation to the nuclear membranes.[19] Another means of action might be complex formation between the inhibitor and the enzyme−substrate complex, rendering the inhibitors uncompetitive substances (studies ongoing).[20]

A set of 38 additional compounds were selected from the NAT-5 library by visual inspection, with an emphasis on compounds structurally similar to our active molecules (structures are listed in Chart S1 in Supporting Information). The goal was to establish a preliminary SAR for the scaffolds of compounds **1** and **2**. Evaluation in intact PNML revealed that two of these compounds (**54** and **55**) have IC_{50} values of approximately 1 μM (Table 2). They are analogues of molecule **1**. Half of the manually selected compounds reduced 5-LO activity by at least 50% at a concentration of 10 μM (Table S3, Supporting Information). Molecule **54** was shown to be the most potent in this set, reducing 5-LO product formation by more than 80% in intact PMNL at a concentration of 10 μM.

Inhibitors **1** and **53**−**55** (Chart 4) were structurally aligned to obtain an idea of a potential pharmacophore (Figure 2). Perfect overlap of pharmacophore features can be seen for the aromatic ring systems of the mutual scaffold, with identical positions of the ether groups. One may argue, however, whether these oxygen atoms actually possess hydrogen-bonding capability. Tight alignment of the bridging amide bonds is observed as expected. The only discrepancy in the alignment is the slightly shifted position of the thiophene ring of molecule **53**, which can be explained by the longer amide linker, mirrored in a greater IC_{50} value. At this position, the two most potent compounds, **1** and **54**, both possess an unsubstituted aromatic heterocycle. In addition, compounds **1** and **53**−**55** possess an *n*-propoxy residue at the phenyl ring. Replacement by a methoxy group (**18**) abolished the efficacy, indicating that a lipophilic and space-filling residue is a requirement for potent 5-LO inhibition. A dimethylamine group at this position (compounds **15** and **17**) revoked inhibitory effects, presumably by preventing the compound from entering the cell. As a consequence, these substances showed almost no inhibitory effects in intact cells, though they exhibited high activity in the assay with partially purified enzyme.

Regarding structural variations of **2**, a large aromatic residue appears to be preferred, since **6** and **7** are weaker inhibitors compared to **2**. It should be noted that all structural neighbors of compound **2** show stronger inhibitory effects in intact PMNL at 10 μM than in the cell-free assay. We also observed that, at a concentration of 1 μM, compounds **2**−**4** and **8** show stronger inhibition of 5-LO product formation in intact PMNLs, while compounds **5**−**7**, **9**, and **10** show superior performance on the partially purified enzyme.

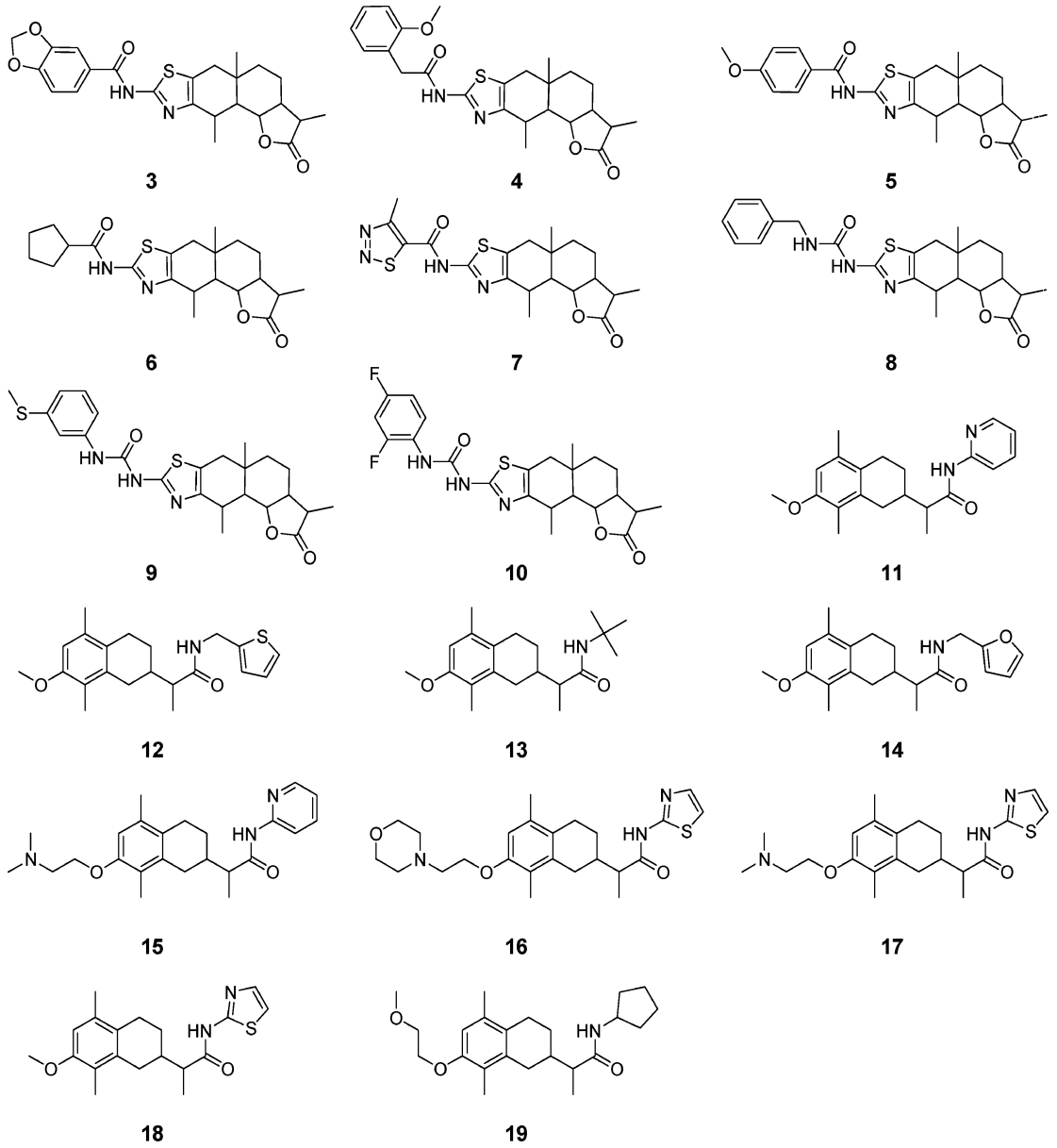
Presently we are unable to assign the new compounds to any of the known 5-LO inhibitor classes, since neither iron-chelating nor redox-active properties of the active compounds are readily apparent. The potency of the compounds is not affected by the redox state of 5-LO, which is a typical feature of non-redox-type 5-LO inhibitors. Initial experiments addressing competitive features with respect to arachidonic acid as 5-LO substrate indicate a noncompetitive or uncompetitive mode of action. Studies are in progress to reveal the basis of the molecular mechanisms of pharmacological interference with 5-LO product synthesis.

Summarizing, our study demonstrates the potential of natural-product derived combinatorial libraries for hit and lead structure finding. A two-step ligand-based virtual screening protocol guided the selection of promising agents with minimal experimental effort. As a result, we were able to identify a novel chemotype that exhibits substantial inhibition of leukotriene synthesis in both intact cells and a cell-free assay.

Materials and Methods

Similarity Searching: (A) SpeedCATS (version 1.1; Schneider Consulting GbR, Oberursel, Germany).[8,12] All molecules were encoded by the topological CATS descriptor.[8] This molecular representation encodes potential two-point pharmacophores in the form of an alignment-free correlation vector.[8,9] For each molecule, the occurrence frequencies of 15 potential pharmacophore point

Chart 3. Virtual Hits[a]



[a] Retrieved by similarity searching in AnalytiCon's NAT-5 compound collection with molecules **1** and **2** as queries.

(PPP; hydrogen-bond donor, hydrogen-bond acceptor, lipophilic, positive ionizable, negative ionizable) pairs were determined for topological distances of 0−9 bonds. This resulted in a 150-dimensional descriptor vector. PPP-pair counts were scaled by PPP-type occurrence. For each of the 43 reference molecules, the 10 most similar molecules were retrieved from the screening pool (AnalytiCon Discovery libraries v.2004: 1298 unique pure natural products in the MEGx collection and 7839 unique natural-product-derived compounds denoted as NATx; AnalytiCon Discovery GmbH, Hermannswerder Haus 17, D-14473 Potsdam, Germany) by use of the Euclidian distance metric.

(B) CATS-*Charge*. This similarity searching approach is based on the correlation vector concept of Gasteiger and co-workers,[21] using estimated three-dimensional conformations and partial atom charges.[9] The three-dimensional Euclidean distances of all atom-pair combinations in one molecule were calculated. Distances within a certain range (0.16 Å) were allocated to the same bin. The charges of the two atoms that form a pair were multiplied to yield a single charge value per pair. Charge values that were assigned to the same bin were summed up. We used 101 bin borders in equidistant steps from 0 to 16 Å. All distances greater than 16 Å were associated with the last bin. The output was a 100-dimensional correlation vector, which characterizes the molecules by means of their partial atom charge distribution. The correlation vector representation was calculated as

$$\mathrm{CV}_d = \sum_{i=1}^{A}\sum_{j=1}^{A}\delta_{ij}(q_i q_j)_d \qquad (1)$$

where d is the atom−atom distance, q_i and q_j are partial atom charges, A is the number of atoms, and δ is the Kronecker delta that equates to 1 if a pair exists and 0 otherwise. The spatial structures for the COBRA database were calculated with the program CORINA (Molecular Networks GmbH, Nägelsbachstrasse 25, D-91052 Erlangen, Germany),[22] and partial atom charges, including hydrogens, were assigned by use of the PETRA software (Molecular Networks GmbH, Nägelsbachstrasse 25, D-91052 Erlangen, Germany).

(C) CATS-*TripleCharge*. The difference between the CATS-*Charge* and the CATS-*TripleCharge* descriptor lies in atom pair counting. While with the CATS-*Charge* descriptor all atom pairs within a certain distance are collected in the same bin, the CATS-*TripleCharge* descriptor differentiates between charge combinations

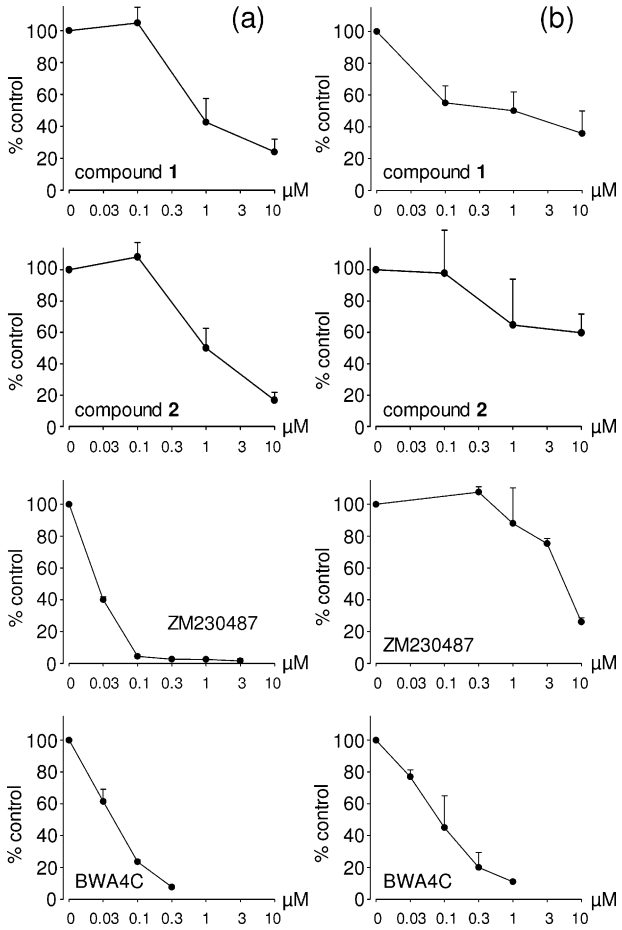


Figure 1. Inhibition of 5-LO product formation by compounds **1** and **2**, positive controls ZM230487 and BWA4C in intact PMNL (a), and of partially purified 5-LO enzyme (b). Error bars give the standard error of the mean.

Table 1. Inhibition of 5-LO Product Formation by Test Compounds in Intact PMNL and of Partially Purified 5-LO Enzyme[a]

	remaining 5-LO activity (%) in intact PMNL		remaining activity (%) of partially purified 5-LO	
no.	@1 μM	@10 μM	@1 μM	@10 μM
1	42 ± 15	24 ± 8	50 ± 12	36 ± 14
2	50 ± 12	16 ± 5	65 ± 29	60 ± 12
3	52 ± 10	30 ± 6	67 ± 14	96 ± 18
4	66 ± 7	26 ± 4	87 ± 21	86 ± 48
5	78 ± 7	29 ± 4	57 ± 7	78 ± 24
6	97 ± 8	33 ± 6	66 ± 10	52 ± 9
7	88 ± 13	50 ± 6	87 ± 25	88 ± 13
8	65 ± 10	14 ± 1	78 ± 19	54 ± 14
9	72 ± 7	19 ± 1	55 ± 18	67 ± 13
10	92 ± 5	28 ± 5	46 ± 12	52 ± 14
11	86 ± 16	10 ± 3	54 ± 9	42 ± 4
12	87 ± 3	41 ± 15	64 ± 17	64 ± 18
13	104 ± 6	56 ± 14	55 ± 18	42 ± 7
14	104 ± 9	85 ± 12	47 ± 15	41 ± 11
15	106 ± 2	116 ± 1	56 ± 7	73 ± 13
16	100 ± 2	70 ± 25	74 ± 19	62 ± 14
17	111 ± 12	78 ± 11	55 ± 5	65 ± 14
18	84 ± 14	66 ± 29	73 ± 15	56 ± 18
19	86 ± 6	53 ± 9	67 ± 30	56 ± 9

[a] Mean values and standard error estimates are given.

++, −−, and +− . So no information is lost by summing up different atom pairs (++/−−, +−) in one bin. Again 101 bin borders covering distances of up to 16 Å were used, with a resulting dimensionality of 300 bins. A related approach was followed by Selzer and Ertl[23] with the aim of classifying G protein-coupled receptor ligands.

Table 2. Determination of IC_{50} Values of Selected Compounds

compd	IC_{50}/intact PMNL (μM)	IC_{50}/partially purified 5-LO (μM)
1	0.8 ± 2	1 ± 10
2	0.9 ± 2	>10
3	2 ± 3	>10
4	4 ± 1	>10
5	6 ± 1	>10
6	8 ± 1	10 ± 5
7	10 ± 2	>10
8	3 ± 1	>10
9	4 ± 1	~1
10	7 ± 1	0.6 ± 0.5
11	5 ± 1	0.8 ± 0.2
12	8 ± 3	>10
13	>10	4 ± 6
14	>10	0.9 ± 10
17	>10	~1
19	>10	>10
53	8 ± 2	3 ± 3
54	0.9 ± 1	6 ± 3
55	1 ± 1	0.8 ± 2

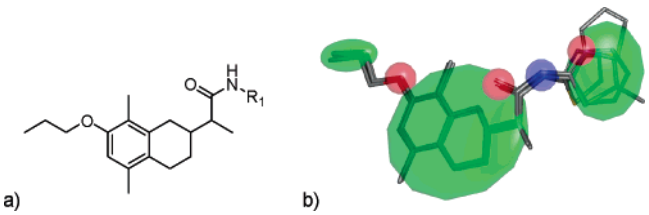
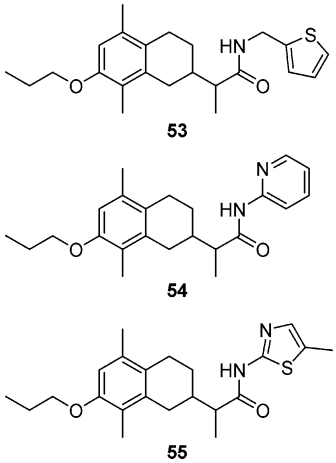


Figure 2. Scaffold (a) and spatial alignment (b) of active molecules **1**, **53**, **54**, and **55** with mutual potential pharmacophore points highlighted. Green shapes represent lipophilic features, red ones show potential hydrogen-bond acceptors, and blue indicates potential hydrogen-bond donor sites. R_1 represents an aromatic ring system.

Chart 4. Potent Manually Selected Compounds[a] That Were Used for Structural Alignment[b]



[a] From the AnalytiCon NAT-5 library. [b]Cf. Figure 2.

(D) MACCS Keys. The 166 public MACCS keys[11] were calculated using the software suite MOE 2004.03 (Chemical Computing Group Inc., Montreal, Canada). The MOE data were transformed into a bit string, a similarity search was performed with the Tanimoto coefficient T (eq 2),[24] and database molecules were ranked according to T.

$$T = \frac{c}{a + b - c} \tag{2}$$

a are the bits set to "1" in molecule A, b are the bits set to "1" in molecule B, and c are the "1" bits common to both molecules. The value of T ranges from 0 to 1. A value of 1 indicates maximal pairwise similarity between the molecular bitstring representations.

Three-Dimensional Pharmacophore Modeling. Three-dimensional conformations and an initial rigid alignment were obtained from MOE (v.2005, Chemical Computing Group Inc., Montreal, Canada). The final alignment was adjusted manually. An extended version of SQUID[25] was used for visualization of pharmacophore features (Y. Tanrikulu et al., unpublished). Potential pharmacophore points (PPPs) are visualized as ellipsoids representing Gaussian feature densities. Features were clustered into PPPs by use of the following cluster radii: lipophilic PPPs, 2 Å; hydrogen-bond donor PPPs, 2 Å; and hydrogen-bond acceptor PPPs, 2 Å. Visualization was performed with PyMOL (The PyMOL Molecular Graphics System, DeLano Scientific, San Carlos, CA).

Assay Systems: (A) Materials. Arachidonic acid and calcium ionophore A23187 were from Sigma (Deisenhofen, Germany). HPLC solvents were from Merck (Darmstadt, Germany).

(B) Cell Preparation. Human PMNL were freshly isolated from leukocyte concentrates obtained at St. Markus Hospital (Frankfurt, Germany). In brief, venous blood was taken from healthy adult donors and leukocyte concentrates were prepared by centrifugation at 4000*g* for 20 min at 20 °C. PMNL were immediately isolated by dextran sedimentation, centrifugation on Nycoprep cushions (PAA Laboratories, Linz, Austria), and hypotonic lysis of erythrocytes as described previously.[26] Cells were finally resuspended in phosphate-buffered saline, pH 7.4 (PBS), containing 1 mg/mL glucose and 1 mM $CaCl_2$ (PGC buffer) (purity >96−97%).

(C) Expression and Purification of 5-LO Protein in *Escherichia coli.* *E. coli* MV1190 was transformed with pT3-5LO plasmid, and recombinant 5-LO protein was expressed at 27 °C as described.[27] For purification of 5-LO protein, *E. coli* cells were lysed by incubation in 50 mM triethanolamine hydrochloride, pH 8.0, 5 mM ethylenediaminetetraacetic acid (EDTA), soybean trypsin inhibitor (60 μg/mL), 1 mM phenylmethanesulfonyl fluoride (PMSF), and lysozyme (500 μg/mL); homogenized by sonication (3 × 15 s); and centrifuged at 19000*g* for 15 min. Proteins including 5-LO were precipitated with 50% saturated ammonium sulfate during stirring on ice for 60 min. The precipitate was collected by centrifugation at 16000*g* for 25 min and the pellet was resuspended in 20 mL of PBS containing 1 mM EDTA and 1 mM PMSF. The resuspended 16000*g* precipitate from *E. coli* was centrifuged at 100000*g* for 70 min at 4 °C, and the 100000*g* supernatant (S100) was applied to an ATP−agarose column. The column was eluted as described previously.[28] Purified 5-LO was immediately used for 5-LO activity assays. Each compound was tested at least three times, and the mean and standard error of the mean were determined.

(D) Determination of 5-LO Product Formation in Intact Cells. For assays of intact cells, 5×10^6 freshly isolated PMNL were resuspended in 1 mL of PGC buffer. After preincubation with the test compounds for 15 min at room temperature, 5-LO product formation was started by addition of 2.5 μM calcium ionophore A23187, plus 20 μM exogenous arachidonic acid. After 10 min at 37 °C, the reaction was stopped with 1 mL of methanol, and 30 μL of 1 N HCl, 200 ng of prostaglandin B_1, and 500 μL of PBS were added. Formed 5-LO metabolites were extracted and analyzed by HPLC as described.[28] 5-LO product formation is expressed as nanograms of 5-LO products per 10^6 cells, which includes LTB_4 and its all-trans isomers, 5(*S*),12(*S*)-dihydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid [5(S),12(S)-diHETE], and 5(*S*)-hydro(pero)-xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid [5-H(p)ETE]. Cysteinyl leukotrienes (LTC_4, D_4, and E_4) were not detected, and oxidation products of LTB_4 were not determined. Each compound was tested at least three times, and the mean and standard error of the mean were determined.

(E) Determination of 5-LO Product Formation in Cell-Free Systems. For determination of the activity of partially purified 5-LO from *E. coli*, 5-LO (corresponding to 0.5 μg of 5-LO protein) was added to 1 mL of a 5-LO reaction mix (PBS, pH 7.4, 1 mM EDTA, and 1 mM ATP). Samples were preincubated with the test compounds. After 5−10 min at 4 °C, samples were prewarmed for 30 s at 37 °C, and 2 mM $CaCl_2$ and 20 μM arachidonic acid were added to start 5-LO product formation. The reaction was stopped after 10 min at 37 °C by addition of 1 mL of ice-cold methanol, and the formed metabolites were analyzed by HPLC as described for intact cells (vide supra). Each compound was tested at least three times, and the mean and standard error of the mean were determined.

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Supporting Information Available: Details on virtual screening results and compound synthesis. This material is available free of charge via the Internet at http://pubs.acs.org.

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