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Scaffold-Hopping Cascade Yields Potent Inhibitors of 5-Lipoxygenase

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5-lipoxygenase (5-LO) is a validated drug target for the treatment of inflammation and allergic reactions as this enzyme is involved in catalyzing the conversion of arachidonic acid into leukotrienes.[1] Inhibitors with dual activity towards both 5-LO and cyclooxygenase (COX), such as Licofelone,[2] have been described as potent analgesic, anti-inflammatory and antiasthmatic agents lacking the gastrointestinal side effects seen with selective COX inhibitors.[3] More recently, dual 5-LO/COX-2 inhibitors have been suggested as potential anticancer drugs.[4] In this study, ligand-based virtual screening methods were used in an iterative fashion to identify new inhibitors of 5-LO product formation. The study consisted of four subsequent cycles of virtual screening, including 3D- and 2D-based methods and substructure searching, as well as biochemical testing. The iterative steps led to the discovery of a pyridine–imidazole-based lead structure series with nanomolar inhibitory activity in a cellular assay, demonstrating the applicability of advanced virtual screening techniques for designing small, focused, screening libraries that yield high hit rates in cell-based assays.

We selected 11 dual 5-LO/COX reference inhibitors from the literature (Supporting Information, figure S1),[5] with the aim to explore the ability of ligand-based virtual screening methods to retrieve isofunctional chemotypes with different backbone architecture ("scaffold-hopping")[6] from a large compound collection. The first step involved a broad, virtual screening process following the protocol outlined in Figure 1. For each of the 11 queries, two alignment-free similarity searches were performed in the Asinex Gold (November 2005: 231812 compounds) and Platinum (132250 compounds) collections (Asinex Ltd., Moscow, Russia) using the "Charge3D"[7,8] and "TripleCharge3D"[7,9] methods.

Briefly, "Charge3D" is an implementation of the correlation vector approach developed by Gasteiger and co-workers.[8] The method compares two molecules based on their three-dimensional distribution of partial atom charges: Euclidian distances

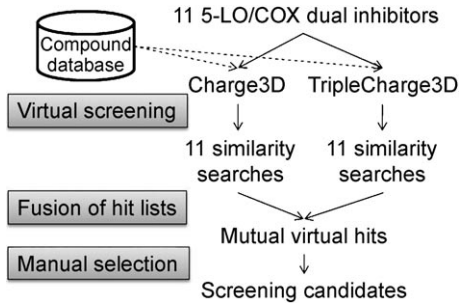

Figure 1. Flowchart of the first virtual screening round.

of all atom-pair combinations in one molecule are calculated (distances within a certain range are allocated to the same bin), and the charge values of the two atoms that form a pair are multiplied to yield a single value per atom-pair (charge values that were assigned to the same bin were added). Equation (1) describes the autocorrelation vector (*CV*) calculation used by "Charge3D", where d is the distance in Å, q_i and q_j are partial atomic charges, A is the number of atoms in a molecule and δ defines the Kronecker delta (1 if a given atom pair exist, 0 otherwise). "TripleCharge3D" is an extension of this technique but makes a distinction between atom-pair types according to charge signs ($++$, $+-$, $--$).

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

For descriptor calculations, a single, low energy conformer was generated for each molecule using CORINA, and partial atomic charges were computed with PETRA (both from Molecular Networks GmbH, Erlangen, Germany). While understanding that, by calculating only a single conformer, the biologically active conformation might not be generated, it has been shown previously that using only one conformer for alignment-free methods can be sufficient.[10]

The ten top-ranking candidate compounds of each of the 22 virtual screens were pooled and compounds detected by both methods were selected for in vitro activity testing (compounds **1**–**11**). A striking feature of this small candidate collection is the dominance of thiazole-based compounds.

Determination of the in vitro activities of compounds **1**–**11** was performed in a cell-based assay designed to measure the inhibition of 5-LO product formation in intact polymorphonuclear leukocytes (PMNL).[7] We deliberately used a whole cell assay for the reason to see whether virtual screening can cope with intracellular bioavailability (in particular membrane permeation) without explicitly predicting this property. Seven out of the 11 compounds tested exhibit low to medium micromolar activity, with compounds **4**, **7**, **8** and **10** being most potent

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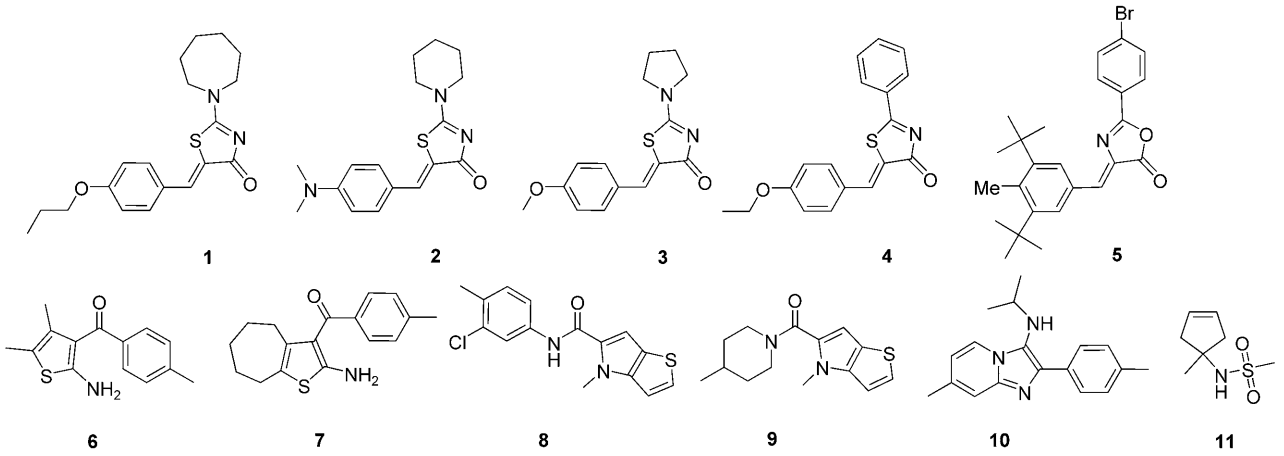


(Table 1). Each of these molecules contains a different scaffold, which clearly demonstrates successful scaffold-hops and validates ligand-based virtual screening for cellular assays.

Table 1. 5-LO inhibitory activities of virtual screening hits in a whole-cell assay.[a]

Compound	IC_{50} [μM][b]	Compound	IC_{50} [μM][b]
1	12±2	**12**	1.3±1
2	20±4	**13**	0.9±0.5
3	inactive	**14**	0.6±0.5
4	2±1	**15**	1.5±0.6
5	inactive	**16**	1.5±0.9
6	15±4	**17**	1.3±0.6
7	3±1	**18**	<3
8	4±1	**19**	inactive
9	inactive	**20**	inactive
10	6±1	**21**	inactive
11	inactive	**22**	15±7
		23	12±9

[a] Whole-cell assay carried out using PMNL cells. [b] Values given are a mean of three experiments with the standard error.

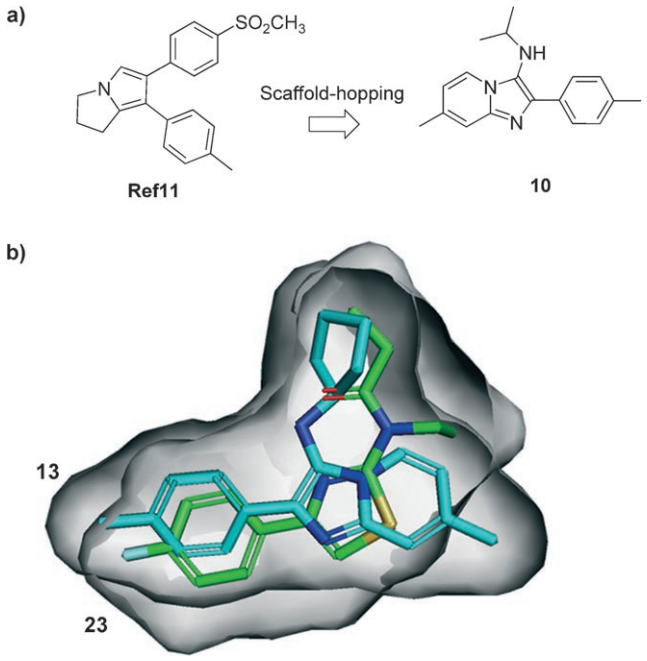
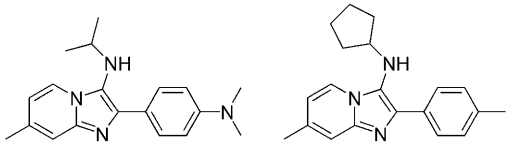


Figure 2. a) Scaffold-hop during the first virtual screening round from the known dual 5-LO/COX inhibitor **Ref11** to compound **10**; b) Shape- and pharmacophore-based alignment of compounds **13** and **23** (scaffold-hop during the fourth virtual screening round).

Compounds **1**–**11** were also tested for COX-2 inhibition in intact Mono Mac 6 (MM6) cells.[11] In this assay, only compound **10** exhibited weak activity (IC_{50} = 70±20 μM). It was found by both virtual screening methods as a relative of the pyrrolizine-derived COX/5-LO reference inhibitor Ref11 (COX-2, IC_{50} = 5 nM; 5-LO, IC_{50} = 10 μM)[12] (Figure 2a). Most surprisingly, however, is the observation that although 5-LO/COX dual inhibitors served as queries for virtual screening, the 5-LO inhibitor pharmacophore is prevalent among the active compounds identified. This implies that 5-LO accepts a broader range of substrate chemotypes compared with COX-2, and the virtual screening tools captured the 5-LO pharmacophore more stringently than the COX-2 ligand pattern.

A similarity search was performed on compound **10** with MOE (version 2006.08, Chemical Computing Group Ltd., Montreal, Canada) using MACCS substructure keys.[13] This involved the generation of MACCS keys from a given set of 166 predefined substructures. A bit is set whenever one of these substructures is present in a molecule. Bitstrings of the query and the database compound were compared using the Tanimoto coefficient,[14] with values ranging from 0 to 1, where 1 indicates bitstring identity. Again, the Asinex Gold compound collection was screened, and compounds with a Tanimoto coefficient >0.85 (indicating high structural similarity to derivative **10**)[14] were identified. From this list, twelve commercially available compounds were manually selected and tested in whole-cell assays.

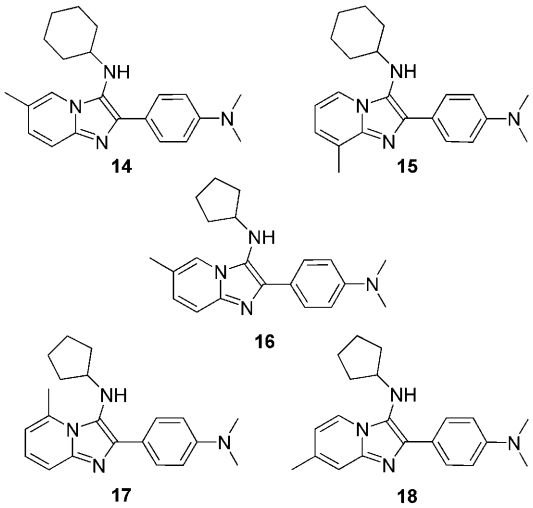
Among these pyridine–imidazoles, derivatives **12** and **13** were most potent, with **13** inhibiting cellular 5-LO product formation in the nanomolar range (Table 1). These results are in agreement with the data obtained from the first virtual screen and further support this scaffold as a potential lead substructure. Compound **12** was also identified in the first virtual



12 13

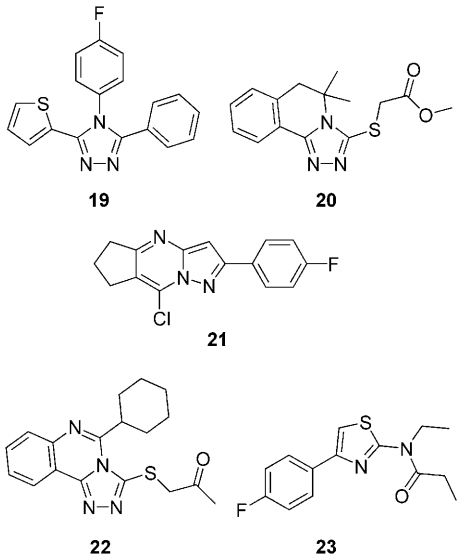
screen with "TripleCharge3D", in the first percentage of the screened database (1323 compounds) at position 48 as a close relative of Ref11. Notably, compound **13** also had a high similarity rank to Ref11 using both "TripleCharge3D" (4th) and "Charge3D" (21st) in the first virtual screening round.

In a third selection round, we examined the influence of substitutions at the pyridine–imidazole ring on 5-LO activity, while retaining the phenyl-dimethylamino-motif. Five compounds were picked from the Asinex Gold collection with compounds **12** and **13** as queries for substructure searching. All five compounds (**14**–**18**) are effective inhibitors of 5-LO product formation, with analogue **14** as the most potent (IC_{50} = 0.6 ± 0.5 μM) (Table 1). The position of the methyl group on the imidazole ring does not significantly affect ligand potency. Equally, the size of the aliphatic ring had no observable effect on the activity. In future studies, it might be worthwhile increasing the volume of this lipophilic substituent and measure its effect.



14 15 16 17 18

Finally, in a fourth virtual screening round, we attempted to find scaffold replacements for the pyridine–imidazole structure using compound **13** as a reference. The shape- and "fuzzy" pharmacophore-based technique SQUIRREL[15] was applied to find candidates in the Asinex Platinum compound collection. SQUIRREL decomposes the molecular surface into patches of equal curvature, which are used to align the underlying structures based on their shape. The quality of the alignments was evaluated by the LIQUID pharmacophore scoring function,[16] which employs multivariate Gaussian distributions of potential pharmacophoric points to calculate the score of the pharmacophore overlap. Five compounds (**19**–**23**) were selected from the results and tested in whole-cell assays. Two compounds (**22** and **23**), containing different scaffolds, showed modest activity (Table 1). Compound **23** was also found by "TripleCharge3D" in the first screening round (35th), with the orally active dual inhibitor RWJ-63556 (COX-2, IC_{50} = 1.86 μM; 5-LO, IC_{50} = 0.13 μM)[17] as the query (Supporting Information, figure S1[5]).



19 20 21 22 23

Although only moderately active hits were obtained in the final screening round, notably, compound **23** contains a similar scaffold to that seen in compounds **1**–**4**, suggesting shape resemblance between pyridine–imidazole- and thiazole-based 5-LO inhibitors (Figure 2b). Thiazolidinones are known 5-LO inhibitors; in 1992 Unangst et al. reported dual 5-LO/COX inhibition by (*Z*)-5-[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]-methylene]-2-imino-4-thiazolidinone.[18] Recently, Geronikaki et al. identified a series of 2-thiazolylimino-5-arylidene-4-thiazolidinones with moderate activity against dual soybean lipoxygenase/COX-1.[19] The results described corroborate this general chemotype for 5-LO inhibition. Notably, the (*Z*)-5-benzylidene-2-phenylthiazol-4(5*H*)-one (**4**) identified in this virtual screen was inactive against COX-2. It is possible that the 2-phenyl substituent in compounds **1**–**4** is responsible for the selective 5-LO activity; to our knowledge, this moiety has not been described as a 5-LO/COX dual inhibitor.

In conclusion, our multistep virtual screening procedure demonstrated that a single round of pharmacophore-based compound ranking is insufficient to identify potent hits, as determined by biological testing. Hits with increased potency can be retrieved by structure-based similarity searching using the highest ranked hits from the previous screen as queries (compounds **12**, **13**). A combination of 3D- and 2D-based similarity searching methods, together with substructure matching, resulted in several potent hits. Subsequent pharmacophore-based virtual screening (fourth cycle in this study) did not lead to the identification of more potent compounds, and instead, additional scaffold-hops were observed. For the purpose of rapid lead structure identification, we have shown that a two-step virtual screening process with a "fuzzy" (more abstract) scoring function in the first cycle, and a substructure-based similarity search in the second round results in the identifica-

tion of potent hits. Biological validation after each cycle is also a key element that guides the virtual screening process.

Experimental Section

Materials: Arachidonic acid and calcium ionophore A23187 were purchase from Sigma–Aldrich (Deisenhofen, Germany). HPLC solvents were obtained from Merck (Darmstadt, Germany).

Cell preparation: Human polymorphonuclear leukocytes (PMNL) were freshly isolated from leukocyte concentrates. 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 isolated by dextrane sedimentation, centrifugation on Nycoprep cushions (PAA Laboratories, Linz, Austria), and hypotonic lysis of erythrocytes as previously described.[20] Cells were resuspended in phosphate-buffered saline (PBS), pH 7.4, containing 1 mg mL^{-1} glucose and 1 mM $CaCl_2$ (PGC buffer) (purity > 96–97 %).

Determination of 5-lipoxygenase product formation in intact cells: For whole-cell assays. freshly isolated PMNL (5×10^6) were resuspended in PGC buffer (1 mL). After pre-incubation with the test compounds for 15 min at RT, 5-LO product formation was stimulated by the addition of calcium ionophore A23187 (2.5 μM) and exogenous arachidonic acid (20 μM). After 10 min at 37 °C, the reaction was stopped with the addition of methanol (1 mL). HCl (30 μL, 1 N), prostaglandin B1 (200 ng) and PBS (500 μL) were added and the 5-LO metabolites were extracted and analyzed by HPLC as described in the literature.[11] 5-LO product formation was determined as nanograms of 5-LO products per 10^6 cells, which includes Leukotriene B4 (LTB4) and its all-*trans* isomers: 5(*S*),12(*S*)-diHETE (5(*S*),12(*S*)-dihydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid) and 5-H(p)ETE 5 ((*S*)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid). Each compound was tested three times, and the mean and standard error of the mean were calculated.

Cyclooxygenase 2 (COX-2) assay: Human monocytic Mono Mac 6 (MM6) cells were differentiated with transforming growth factor beta (TGFβ, 1 ng mL^{-1}) and calcitriol (50 nM) for 96 h as described.[11,21] Lipopolysaccharide (100 ng mL^{-1}) was added to induce COX-2 expression 6 h prior to harvest. The harvested cells were washed twice, resuspended in PGC buffer (5×10^6 cells mL^{-1}), preincubated with the test compounds for 15 min at 37 °C, and then incubated with arachidonic acid (30 μM) for 15 min at 37 °C. The reaction was stopped by cooling on ice for 10 min. Cells were centrifuged (300 g, 5 min, 4 °C) and the amount of 6-keto-$PGF_{1\alpha}$ released was assessed by ELISA using a monoclonal antibody against 6-keto $PGF_{1\alpha}$ according to the protocol described by Yamamoto and coworkers.[22]

Computational methods: Shape-based molecular alignment during the fourth virtual screening round was performed with the SQUIRREL software.[15] Single conformations were obtained using CORINA version 3.2 (Molecular Networks GmbH, Erlangen, Germany).

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