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Novel human lipoxygenase inhibitors discovered using virtual screening with homology models

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Abstract

We report the discovery of new, low micromolar, small molecule inhibitors of human platelet-type 12- and reticulocyte 15-lipoxygenase-1 (12-hLO and 15-hLO) using structure-based methods. Specifically, we created homology models of 12-hLO and 15-hLO, based on the structure of rabbit 15-lipoxygenase, for *in silico* screening of a large compound library followed by *in vitro* screening of 20 top scoring molecules. Eight of these compounds inhibited either 12- or 15-human lipoxygenase with lower than 100 μM affinity. Of these, we obtained IC_{50} values for the three best inhibitors, all of which displayed low micromolar inhibition. One compound showed specificity for 15-hLO versus 12-hLO; however, a selective inhibitor for 12-hLO was not identified. As a control we screened 20 randomly selected compounds, of which none showed low micromolar inhibition. The new low-micromolar inhibitors appear to be suitable as leads for further inhibitor development efforts against 12-hLO and 15-hLO, based on the fact their size and chemical properties are appropriate to classify them as drug-like compounds. The models of these protein-inhibitor complexes suggest strategies for future development of selective lipoxygenase inhibitors.

Introduction

Lipoxygenases are a class of non-heme, iron containing enzymes that catalyze the incorporation of molecular oxygen into 1,4,-*cis,cis*-pentadiene containing fatty acids (*e.g.* linoleic and arachidonic acids) to form hydroperoxide products[1-4]. Lipoxygenases are the first committed step in a cascade of metabolic pathways that are implicated in the onset of cancers, asthma and heart disease, making them candidates for inhibitory pharmaceutical therapy[5-7]. The human isozymes, 5-, 12- and 15-lipoxygenase, are associated with different disease states, which suggests that selective inhibition may be important in targeting them for therapeutic purposes[8-10]. Recent discovery efforts of lipoxygenase inhibitors[11-13] have focused especially on structure-activity relationships[14-22] and natural product isolation[23-31]. In a very recent report, pharmacophore virtual screening methods have yielded novel inhibitors with selectivity for rabbit 15-lipoxygenase[32]. One selective human 5-lipoxygenase inhibitor, Zileuton[21], has been approved by the FDA for the treatment of asthma[33].

We report the discovery of new, low micromolar, small molecule inhibitors of human platelet-type 12-lipoxygenase (12-hLO) and human reticulocylte 15-lipoxygenase-1 (15-hLO) using structure-based, *in silico* methods and *in vitro* screening. Prior to this work, structure-based methods have not played a major role in the discovery of lipoxygenase inhibitors, because experimental structures of the human lipoxygenases do not exist. Here, we create homology models of 12-hLO and 15-hLO, based on the structure of rabbit reticulocyte 15-lipoxygenase (15-rLO)[34], followed by docking of a large compound library and *in vitro* screening of the top scoring molecules.

Although docking using comparative models is more challenging and less successful than docking to crystallographic structures, a recent review by Jacobson and Sali[35] describes efforts dating back to at least 1993 in which comparative protein models have been successfully used to aid inhibitor discovery. Some of the proteins successfully targeted using docking against comparative models include cysteine proteases in several parasites[36-39], matriptase[40], Bcl-2[41], retinoic acid receptor[42], DHFR in *T. cruz*[43], human CK2[44], and CDK4[45]. Despite these successes, however, the role of comparative models in structure-guided drug design remains small. A few studies including those by McGovern and Shoichet[46], Diller and Lee[47] and Oshiro *et al.*[48] have attempted to quantify the accuracy of docking to comparative models by evaluating the ability to enrich known inhibitors relative to large databases of "decoy" ligands. The success of docking against homology models varies significantly from case to case in these studies, but in general is somewhat poorer than docking against crystal structures.

Homology models of lipoxygenase enzymes have been used in several previous studies for purposes other than inhibitor discovery[49-51]. Specifically, these studies have used homology models, sometimes in combination with docking methods, to propose models for substrate binding and specificity in various lipoxygenases.

In previous work, we have used docking methods to propose a binding model where linoleic acid binds "carboxylic end first" in the active site of soybean lipoxygenase, which was consistent with experimental mutagenesis studies[52]. In this work, we performed virtual screening of 50,000 drug-like molecules against the active sites of 12-hLO and 15-hLO homology models. The top ten compounds from each virtual screen were then experimentally screened against each enzyme; no filtering of the computational results was performed. Eight of these 20 compounds inhibited either 12-hLO or 15-hLO with lower than 100 μM affinity. Of these, we obtained IC_{50}

values for the three best inhibitors, all of which displayed low micromolar inhibition. One compound shows specificity for 15-hLO versus 12-hLO; however, a selective inhibitor for 12-hLO was not identified. As a control we also screened 20 randomly selected compounds, of which none showed low micromolar inhibition. The new low-micromolar inhibitors generally adhere to Lipinski's rules of five, with average molecular weights of 350, less than five rotatable bonds and an average logP of four. Moreover, these inhibitors are chemically dissimilar from known lipoxygenase inhibitors and provide new scaffolds that may be useful for further development of selective lipoxygenase inhibitors.

Methods

Homology Model Construction. The 12-hLO and 15-hLO homology models were built using the 2.4 Å resolution 15-rLO structure (PDB ID: 1LOX), with the co-crystallized Roche RS7 inhibitor, as a template[53]. This is the only publicly available crystal structure of a mammalian lipoxygenase. It has 80% overall sequence identity to 15-hLO and 61% sequence identity to 12-hLO. The template structure was first refined by the addition of hydrogen atoms not present in the crystal structure, as well as the construction of atoms and residues not resolved in the crystal structure. Residues 201-211 and 601-602 lacked electron density and could not be resolved. In addition, the electron densities of residues 177-188 were ambiguous due to the presence of a 2-fold crystallographic symmetry operator. Construction of the missing segments was carried out in the Jacobson group using their in-house software, Protein Local Optimization Program (PLOP, distributed commercially under the name Prime), which uses loop prediction[54], side chain prediction[55, 56], and energy minimization to construct the missing atoms and residues.

The homology modeling of 12-hLO and 15-hLO was also carried out using PLOP, based on alignments obtained from NCBI-BLAST (blastp)[57]. In brief, the primary structural modifications that must be made during the initial model construction are the closing of chain breaks associated with gaps in the sequence alignment, and side chain optimization (for all residues that are not identical between target and template in the sequence alignment). Chain break closure is accomplished using iterative application of a loop prediction algorithm, which is described in detail elsewhere[54]. After all chain breaks have been closed, side chain optimization[55, 56] and complete energy minimization are performed on all portions of the protein whose coordinates were either not taken from the template at all, or were modified during the model building procedure. The homology modeling procedure uses the OPLS all-atom force field[55, 58, 59] and a Generalized Born solvent model[60, 61] for choosing low-energy structures.

Virtual Screening Procedure. Flexible ligand docking was performed using the Glide (Schrödinger, Inc.) program[62, 63], which uses a modified version of the Chemscore energy function to score the protein-ligand interactions[64]. This docking algorithm has recently been shown to outperform other docking algorithms in its ability to reproduce co-crystallized poses from a set of 69 ligand-receptor complexes from the PDB[65]. Molecules were docked using either the standard precision mode (SP) or the extra precision mode (XP), which uses a more optimized scoring function as well as a more extensive search of ligand confirmations than the SP mode. The resulting poses of the docked compounds were ranked according to their GlideScore.

The modeled structures for 12-hLO and 15-hLO and the 15-rLO crystal structure were prepared for docking by minimizing the energy of each structure with the RS7 inhibitor bound in the active site.

The homology models were built with the active site iron in the active ferric state (Fe^{3+}) with no bound water. The ferrous (Fe^{2+}) form of the enzyme is dominant at equilibrium and is activated by oxidation of one equivalent of the hydroperoxide product [(9*Z*-11*E*)-13-hydroperoxy-9,11-octadecadienoic acid (HPOD)] to the ferric state (Fe^{3+}). The interaction with the substrate, linoleic or arachidonic acid, reduces the iron from the ferric to the ferrous state. We assumed that the putative inhibitors would act as competitive inhibitors, thereby disallowing the entry of the substrate and subsequently not allowing the iron to be reduced.

The databases of small molecules were prepared for docking using the LigPrep (Schrödinger, Inc) ligand preparation software, which generates a minimized conformation of each ligand, and multiple protonation/tautomerization states when appropriate.

Experimental Determination of IC_{50}'s. The experimental screening was performed as previously described[28]. Briefly, the human lipoxygenases were expressed and purified via our standard his-tagged protein protocol[27]. 12-hLO and 15-hLO were then screened for inhibition by monitoring the rate of formation of the conjugated diene products at 234 nm via UV spectroscopy. One-point screens were preformed with 20 μL of a 1 mg/mL solution of inhibitor in DMSO aliquoted into a 2 mL cuvette containing 4 μM of substrate in the appropriate buffer (Hepes pH 8.0 for 12-hLO and Hepes pH 7.5 for 15-hLO) containing 0.01% by weight Triton X-100 to disrupt small molecule aggregates that can lead to false positives[46]. The percent inhibition (%inh) is given as %inh = $(1 - R_I/R_c)$, where R_I is the enzyme rate with the inhibitor present and R_c is the control rate of the enzyme. The control is performed as described above in the absence of inhibitor but with the same volume of DMSO. Initially, one-point screens were performed with 0.01% concentration of inhibitor, in duplicate, to assess the effectiveness of inhibitor. If the inhibitor did not display low potent inhibition, the IC_{50} was estimated with the one-point screen, utilizing the hyperbolic equation, %inh = [I]/([I]+IC_{50}), where [I] is the concentration of the inhibitor and %inh is the percent inhibition, assuming one-hundred percent inhibition at infinite [I]. Compounds that displayed potent inhibition were screened at multiple inhibitor concentrations (2-60 μL of a 0.1 to 1 mg/mL solution in DMSO, depending on inhibitor strength) and fit with a standard hyperbolic equation to determine IC_{50} values and their corresponding error.

Results

As a first test, we confirmed the ability of the docking algorithm to reproduce the co-crystallized pose of the RS7 inhibitor in the 15-rLO crystal structure (Figure 1). The RMS deviation of the RS7 inhibitor from its co-crystallized position is 0.67 Å (docking pose accuracy of better than 2.0 Å RMSD is generally considered acceptable). We also docked the RS7 inhibitor against the 12-hLO and 15-hLO homology models, although we know of no published results showing that it inhibits these enzymes. The RMS deviations of the RS7 inhibitor from its co-crystallized position are 0.90 Å and 1.84 Å when docked against 15-hLO and 12-hLO respectively.

Figure 2 depicts surface representations of the substrate binding sites in the 15-hLO and 12-hLO homology models as well as the 15-rLO template. Selected residues that determine the size of the active site, and thus determine the chemistry performed by the respective enzymes, are depicted. The active sites of the 15-hLO and 15-rLO are roughly the same size but the 12-hLO active site is larger and extends further towards the back of the active site. This is consistent with experimental mutagenesis studies that demonstrated that the 12-hLO active site is larger than that of 15-hLO[66].

Subsequently, a set of 50,000 drug-like compounds, commercially available from ChemBridge ("diversity set"), was docked against the 12-hLO and 15-hLO homology models, using Glide's SP ("standard precision") mode. The top 1000 hits from this initial screen (ranked according to GlideScore) for both the 12-hLO and 15-hLO homology models were then refined by re-docking them using Glide's XP ("extra precision") mode.

The top 10 compounds from this docking protocol against both 12-hLO and 15-hLO were purchased and experimentally screened for their ability to inhibit lipoxygenase activity. All twenty compounds taken from the docking calculations were visually inspected to ensure reasonableness, but none were omitted from experimental testing. All 20 of the top hits formed putative hydrogen bonds to Gln546 and Glu355 in the active site and formed extensive hydrophobic interactions in the rear of the active site.

The results obtained from the experimental screen are shown in Table 1. Of the top ten scoring compounds from the 15-hLO docking calculations, one compound (h15-009) showed non-selective inhibition against both 15- and 12-hLO with IC_{50} values of 9.2 ± 1.4 and 12.3 ± 0.9 μM, respectively. Of the top ten scoring compounds taken from the 12-hLO docking calculation, two exhibited low micromolar inhibition. Compound h12-002 inhibited both 12-hLO and 15-hLO with IC_{50} values of 30.7 ± 6.8 μM and 18.8 ± 4.7 μM, respectively. However, h12-005 was selective against 15-hLO (IC_{50} = 6.8 ± 1.2 μM) and not 12-hLO (IC_{50} > 200 μM). All three of the compounds described above have a basic nitrogen in the central region of the molecule that, if protonated, could form an ion pair with Glu355, as suggested by the docking pose. For example, the amidinium group of h12-005 has a pKa of approximately 6-7, and thus could be protonated. This feature is lacking in most of the other compounds tested, which were less potent or showed no inhibitory activity. Most of these other compounds do contain nitrogens in the central region of the molecule which could hydrogen bond to Glu355, but most of these nitrogens form amide-type bonds in the ligand and thus will not carry a positive charge. These results suggest that compensating this negative charge on Glu355 may be important for designing competitive lipoxygenase inhibitors that occupy that region of the substrate binding site.

Figure 3 shows the h12-005 compound docked into both 12-hLO and 15-hLO. This compound makes the same hydrogen bond contacts in both enzymes, but fills out the hydrophobic end of the binding pocket to a much greater extent in 15-hLO relative to 12-hLO. We believe that the tighter binding in 15-hLO may be due to the favorable van der Waals contacts that form at the bottom of the binding pocket, as well as, potentially, an unfavorable free energy contribution in 12-hLO arising from *not* filling the binding pocket. That is, we believe that the pose of this ligand docked in the homology models provides a *post facto* but reasonable explanation for the observed selectivity of h12-005.

As a control, we also purchased 20 randomly chosen compounds from the ChemBridge library (using a simple computer script employing a random number generator). None of these randomly selected compounds were among the top 1000 compounds from the 12-hLO and 15-hLO docking calculations. No low micromolar inhibitors were identified among the controls, although a few relatively weak inhibitors were identified (Table 2).

Discussion

The primary conclusion of this study is that structure-based virtual screening represents a viable approach to the development of new lipoxygenase inhibitors, despite the fact that no experimental structures are available for human lipoxygenases. Although this is admittedly a relatively small-scale study, with only 20 compounds tested based on the virtual screening results, 3 new low micromolar inhibitors were discovered. These compounds appear to be suitable as leads for further inhibitor development efforts against 12-hLO and 15-hLO, based on the fact their size and chemical properties are generally appropriate to classify them as drug-like compounds (Table 3). This study thus joins a relatively small but growing literature in which homology models have been used in conjunction with virtual screening to successfully identify new enzyme inhibitors.

We wish to underscore two methodological strengths of this study. First, in contrast to many reported applications of virtual screening methods, we did not employ human judgment in selecting compounds from the docking "hit list" for experimental testing. Thus, the successful identification of new inhibitors directly reflects the quality of the homology models and the docking algorithms. Second, as a control study, 20 randomly chosen compounds, from the same library used for the virtual screening, were also tested for inhibition of 12-hLO and 15-hLO. Although this again is clearly a small-scale study, it is nonetheless reassuring that no low-micromolar inhibitors were identified among the randomly selected compounds. We note also that detergent was used in the assay to help disrupt small molecule aggregates that can lead to false positives. Altogether, our results suggest that virtual screening against the homology models is capable of identifying low-micromolar inhibitors at a rate that, in all likelihood, greatly exceeds random compound selection.

Our on-going goal is to identify inhibitors that are both potent and *selective* for specific lipoxygenase enzymes. In the work reported here, we can claim, at best, to be only partially successful in this endeavor. One of the low micromolar inhibitors showed selectivity against 15-hLO over 12-hLO. However, this compound was selected from the virtual screen against the 12-

hLO homology model. Nonetheless, we were able to use our models to retrospectively rationalize why compound h12-005 showed selectivity for 15-hLO. Finally, the models of the docked low-micromolar inhibitors suggest clear strategies for exploiting the differences in binding site volume between 12-hLO and 15-hLO and we remain optimistic that our structure-based approach will ultimately be capable of aiding the discovery of new selective inhibitors, including compounds with selectivity for 12-hLO.

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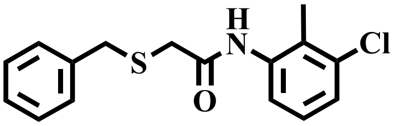
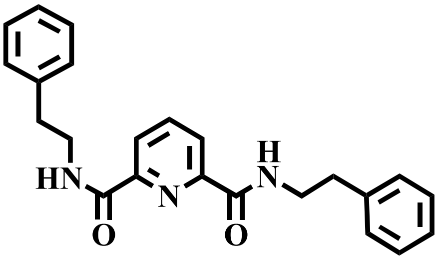
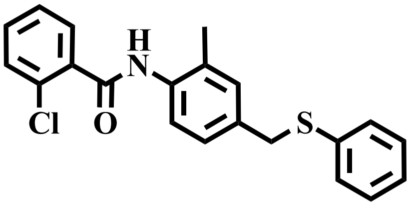
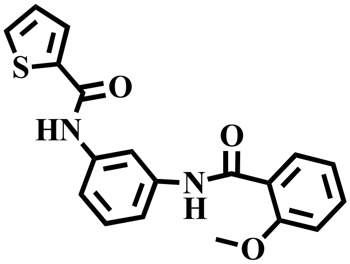
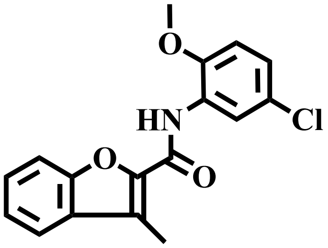
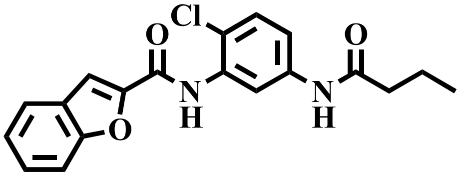
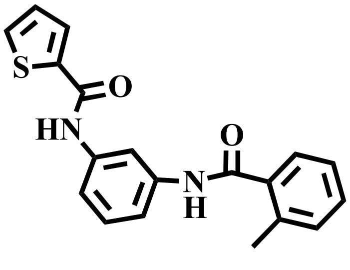
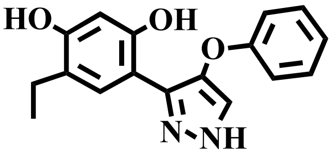
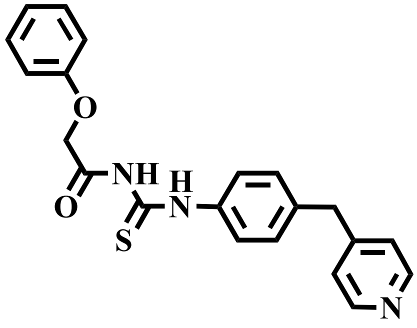
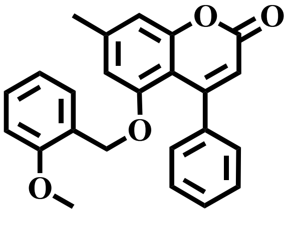
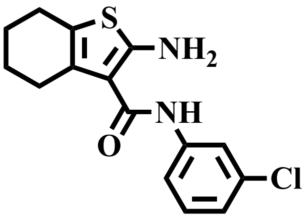
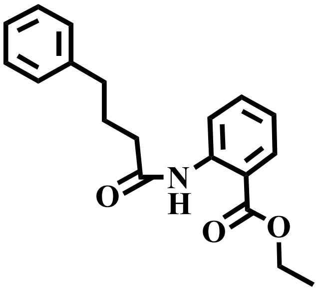
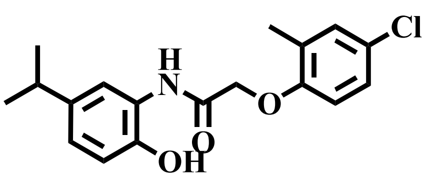
Tables

Table 1. Chemical structures, docking ranks, and IC_{50} values for top scoring compounds in the docking screens against the models of 12-hLO and 15-hLO. Only the top 1000 compounds from the Glide SP results were subjected to re-scoring with Glide XP. Some compounds did not successfully dock into one or both of the models, generally due to a steric clash or other highly unfavorable interaction. The IC_{50} values for the 3 low micromolar inhibitors were determined by fitting the data presented in Supplementary Materials. The IC_{50} values for the remaining molecules were estimated using single-point screens, and categorized as follows: >50 (50-100 μM), >100 (100-200 μM), >200 (200-1000 μM), and >1000 μM.

Chembridge ID Number	ID Number From Text	Structure	SP Rank		XP Rank		IC_{50}	
			15-hLO	12-hLO	15-hLO	12-hLO	15-hLO	12-hLO
6485150	h15-001		708	2949	1	–	>50	>100
5245411	h15-002		107	–	2	–	>100	>200
6738761	h15-004		194	31905	4	–	>100	>200

6712889	h15-005		67	24753	5	–	>50	>100
6671738	h15-006		969	3277	6	–	>100	>100
7510319	h15-007		298	–	7	–	>100	>200
6704363	h15-008		40	12917	8	–	>50	>100
6640337	h15-009		569	4781	9	–	9.2 ± 1.4	12.3 ± 0.9
6615087	h15-010		1435	32049	10	–	>100	>200

								
6942880	h12-001		–	713	–	1	>100	>200
5680672	h12-002		1892	180	–	2	18.8 ± 4.7	30.7 ± 6.8
6862629	h12-003		–	491	–	3	>50	>100
6945303	h12-004		–	609	–	4	>200	>200
6635967	h12-005		1409	301	–	5	6.8 ± 1.2	>200

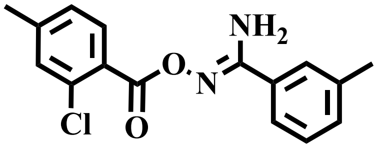
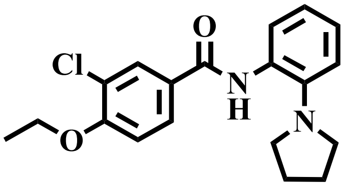
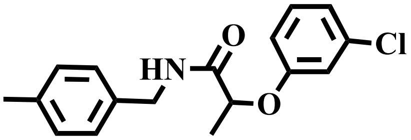
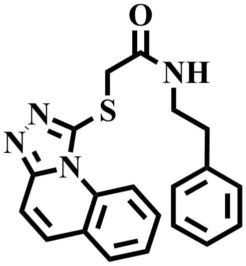
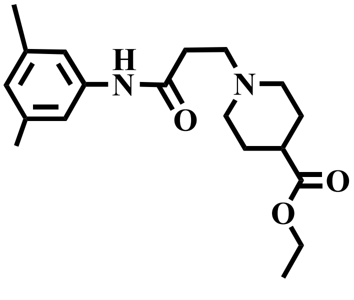
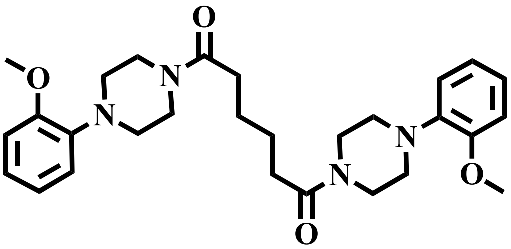
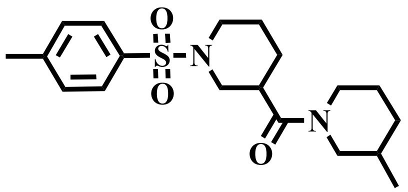
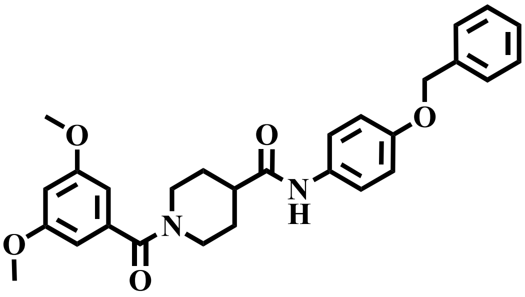
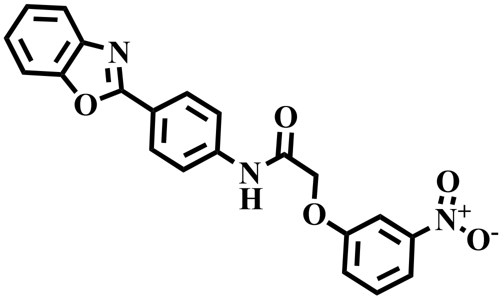
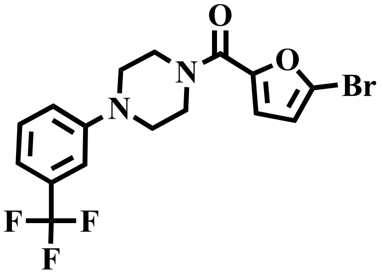
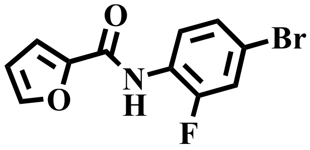
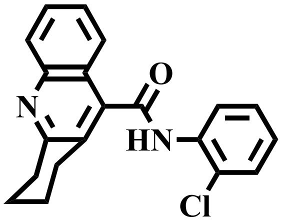
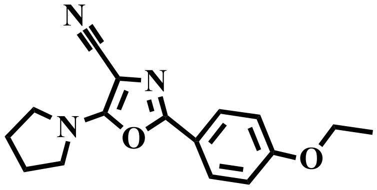
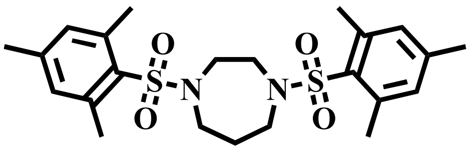
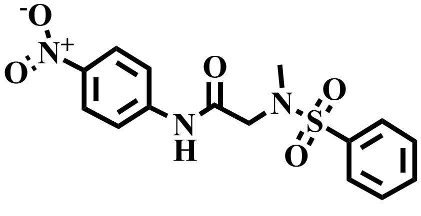
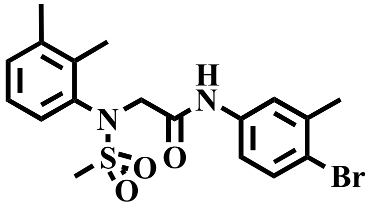
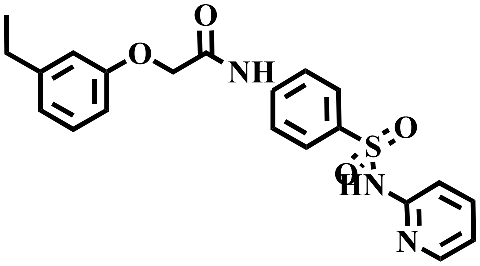
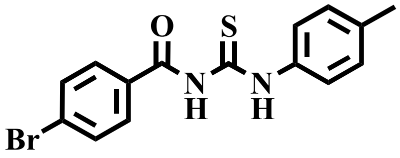
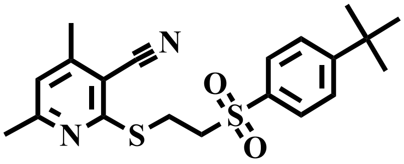
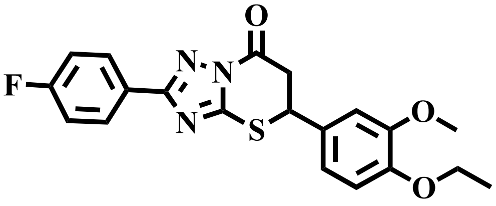
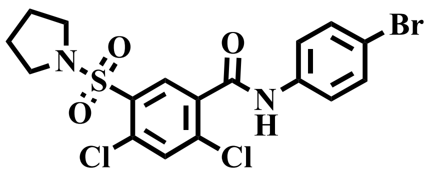
									
7402594	h12-006		–	23094	–	6	>50	>100
7282756	h12-007		–	832	–	7	>100	>200
7383862	h12-008		–	255	–	8	>100	>100
6558319	h12-009		–	709	–	9	>100	>100
7232391	h12-010		–	29698	–	10	>100	>100

Table 2. Structures, ranks from Glide SP docking, and estimated IC$_{50}$ values for the randomly selected compounds. Some compounds did not successfully dock into one or both of the models, generally due to a steric clash or other highly unfavorable interaction. "Agg" refers to compounds that displayed visible aggregation, despite the use of detergent in the assay.

Chembridge ID Number	Structure	SP Rank		IC$_{50}$	
		15-hLO	12-hLO	15-hLO	12-hLO
7176956		–	–	Agg	Agg
6984363		–	30145	>1000	>1000
7376159		–	22483	>100	>1000
7275153		–	25089	>200	>50

					
5666000		6387	15076	>1000	>1000
6943652		–	18545	>1000	>100
7498141		–	10570	>1000	>50
6979008		–	–	>50	>100
7107650		–	21508	>1000	>100
6814786		–	–	Agg	Agg

					
7490554		–	32490	>1000	>1000
6352358		9464	13277	Agg	Agg
7568297		–	31175	>500	>200
6944131		–	–	>50	>1000
6681343		–	–	>200	>1000
6893525		–	1807	>100	>1000

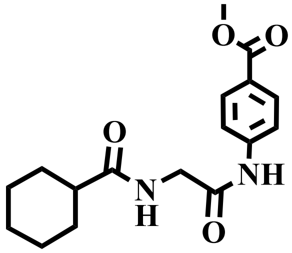
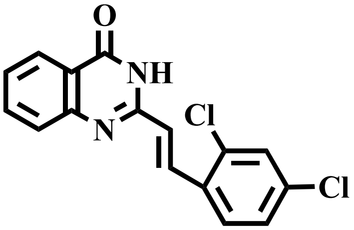
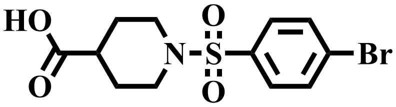
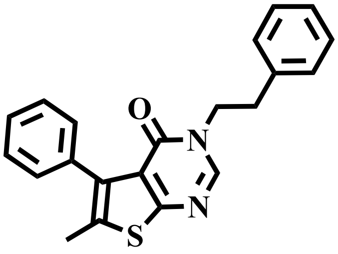
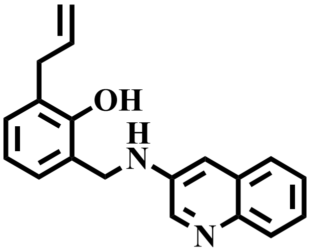
					
7198612		–	–	>50	>1000
6682949		–	2755	>1000	>1000
7502120		–	–	>200	>1000
7406109		–	6362	>1000	>1000

Table 3. Chemical properties of the low micromolar inhibitors.

Chembridge ID	ID Number from Text	Molecular Weight	LogP	Rotatable Bonds	Hbond donors	Hbond acceptors
6635967	h12-005	302.75	5.42	4	1	3
5680672	h12-002	306.81	4.64	2	2	1
6640337	h15-009	296.32	3.93	3	3	4

Figures

Figure 1. Superimposed images of the co-crystallized inhibitor RS7 in the 15-rLO crystal structure (grey), as well as the same inhibitor docked into 15-rLO structure (purple) and the 12-hLO (yellow) and 15-hLO (teal) homology models. The lipoxygenase proteins are not shown, but are structurally superimposed on the 15-rLO crystal structure.

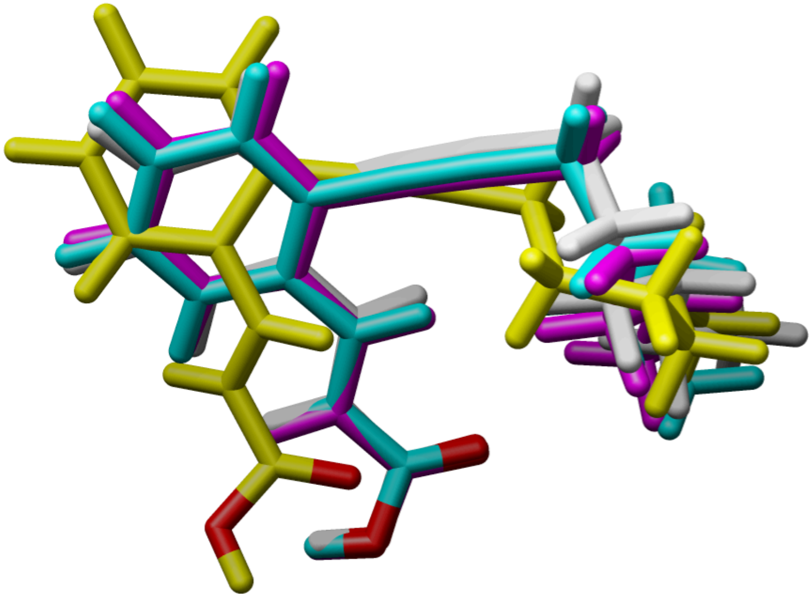


Figure 2. Surface representation of the 15-rLO (a), 15-hLO (b) and 12-hLO (c) active sites with the co-crystallized pose of the RS7 inhibitor shown for reference. The residues shown in wireframe are those that are primarily responsible for determining the size/depth of the active site among the three isozymes (Met and Ile in 15-rLO and 15-hLO; Val and Ala in 12-hLO). The iron is shown in a spacefilling representation. The colors on the surface reflect the identity of the heavy atoms lining the cavity: red for oxygen, blue for nitrogen, and gray for carbon.

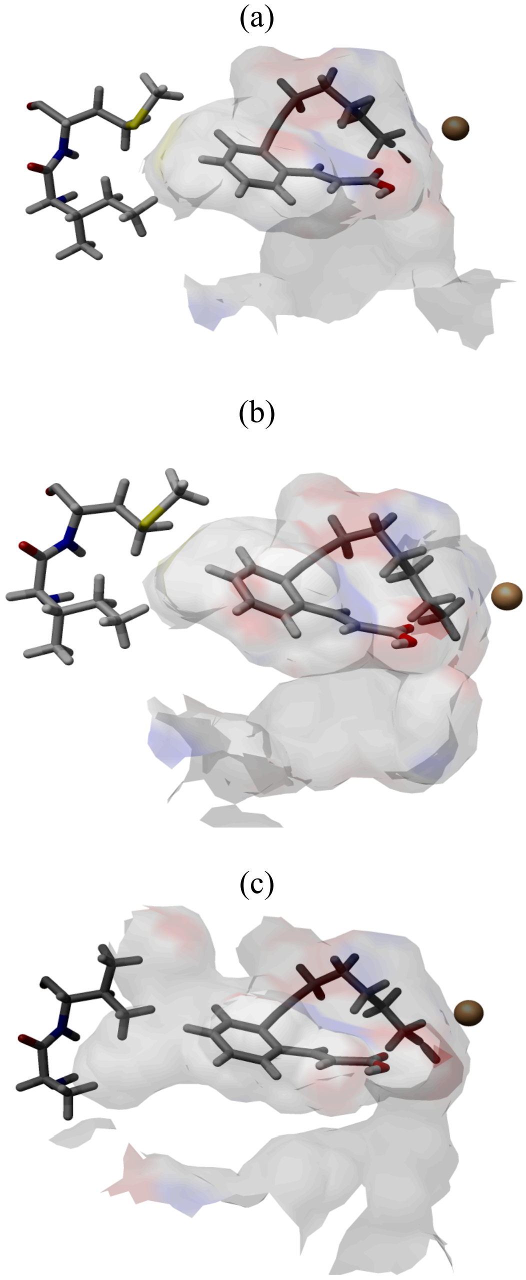
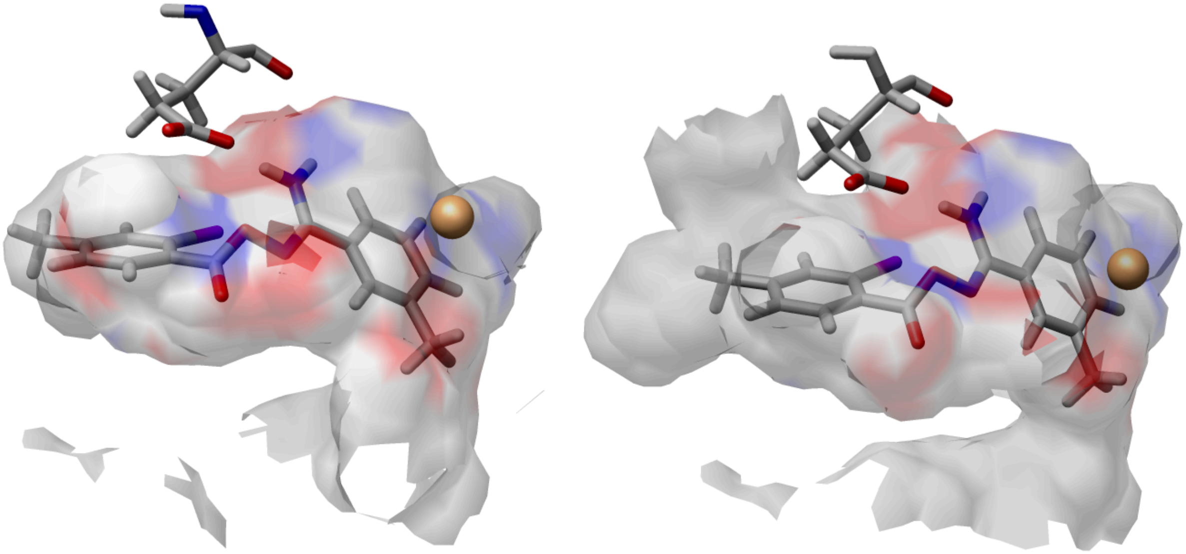


Figure 3. Surface representations of 15-hLO selective compound h12-005 docked to 15-hLO (left) and 12-hLO (right). Glu355 from the receptor is shown in wireframe, because it forms hydrogen bonds to the inhibitor. The iron is shown in a spacefilling representation. The colors on the surface reflect the identity of the heavy atoms lining the cavity: red for oxygen, blue for nitrogen, and gray for carbon. The h12-005 inhibitor fills the binding pocket in 15-hLO much better than in 12-hLO, possibly providing an explanation for its selectivity against 15-hLO.



TOC Graphic