Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

Dynamic Modeling of Human 5-Lipoxygenase–Inhibitor Interactions Helps To Discover Novel Inhibitors

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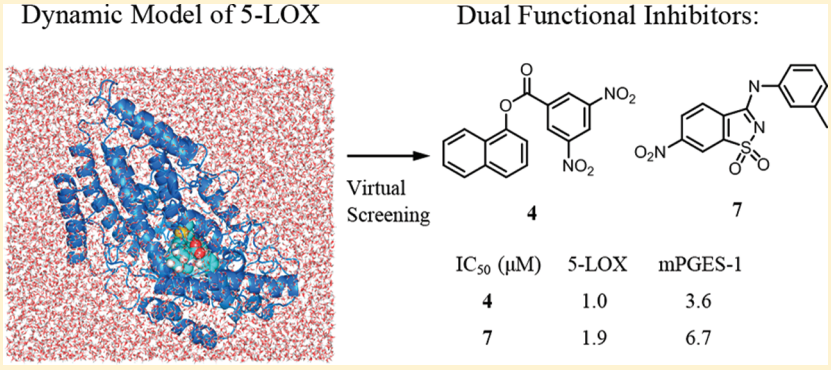
Supporting Information



ABSTRACT: Human 5-lipoxygenase (5-LOX) is one of the key anti-inflammatory drug targets due to its key role in leukotrienes biosynthesis. We have built a model for the active conformation of human 5-LOX using comparative modeling, docking of known inhibitors, and molecular dynamics simulation. Using this model, novel 5-LOX inhibitors were identified by virtual screen. Of the 105 compounds tested in a cell-free assay, 30 have IC_{50} values less than 100 μM and 11 less than 10 μM with the strongest inhibition of 620 nM. Compounds **4**, **7**, and **11** showed strong inhibition activity in the human whole blood (HWB) assay with IC_{50} values of 8.6, 9.7, 8.1 μM, respectively. Moreover, compounds **4** and **7** were also found to inhibit microsomal prostaglandin E synthase (mPGES)-1 with micromolar IC_{50} values, similar to licofelone, a dual functional inhibitor of 5-LOX/mPGES-1. The compounds reported here provide new scaffolds for anti-inflammatory drug design.

INTRODUCTION

Human 5-lipoxygenase (5-LOX) is a validated target for anti-inflammation drug design. It catalyzes the first two reactions in the production of leukotrienes from arachidonic acid (AA): oxygenation of AA to 5(*S*)-hydroperoxy-6-*trans*-8,11,14,9-eicosatetraenoic acid (5-HPETE) and further dehydration to leukotriene A_4 (LTA_4).[1,2] Then LTA_4 is metabolized to other leukotrienes. For example, LTA_4 is converted by LTA_4 hydrolase (LTA_4H) to leukotriene B_4 (LTB_4), an important inflammatory mediator that stimulates neutrophil chemotaxis, aggregation, degranulation, adhesion to endothelial cells, and lysosomal enzyme release.[3] Furthermore, 5-LOX is also discovered to play roles in tumor formation and cancer metastasis and thus is identified as a potential target for anticancer drugs.[4]

Different types of 5-LOX inhibitors have been reported, e.g., redox, iron ligand, and nonredox inhibitors (for review see ref 5), but very few maintain the activity in vivo so far. The only 5-LOX inhibitor on the market is zileuton [*N*-(1-benzo[*b*]thien-2-ylethyl)-*N*-hydroxyurea],[6] which is used for the treatment of asthma (IC_{50} = 0.5−1 μM in stimulated leukocytes). However, weak potency and rapid clearance are the therapeutic drawbacks of zileuton.[7] Some competitive 5-LOX inhibitors have been proved to be orally active, such as methoxytetrahydropyrans ZD-2138 and ZM-230487 and naphthalenic lignan lactones L-697,108 and L-739,010.[5,8] In recent years, multifunctional inhibitors of 5-LOX and other enzymes in the arachidonic acid metabolic network have been paid much attention. Licofelone (ML-3000, 2-[6-(4-chlorophenyl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1*H*-pyrrolizin-5-yl]acetic acid), a compound that inhibits 5-LOX, cyclooxygenase (COX)-1, and microsomal prostaglandin E synthase (mPGES)-1, is undergoing a phase III clinical trial for treating osteoarthritis.[9]

5-LOX belongs to the lipoxygenase family, which catalyzes the hydroperoxidation of polyunsaturated fatty acids (arachidonic acid in animals, linoleic and linolenic acids in plants).[10] All lipoxygenases are homologous in sequence and have the same two-domain structure: an N-terminal β-barrel domain (C2-like or PLAT domain) and a C-terminal catalytic domain (lipoxygenase domain). A catalytic iron atom resides in the C-terminal domain. Before the crystal structure of human 5-LOX

Received: October 14, 2011
Published: March 1, 2012

was reported in 2011,[11] all published drug design studies were based on comparative models built from other lipoxygenases (rabbit 15-LOX,[12] coral 8-LOX,[13] or soybean lipoxygenases[14,15]). In 2008, Choi et al. reinterpreted the crystallographic data of rabbit 15-LOX structure (PDB entry 1LOX)[12] and published a revised model (PDB entry 2P0M).[16] In the revised model, 15-LOX was found in two different conformations: an open model, which has a narrow pocket (220 Å^3) with entrance open to the surface of the protein, and a closed model, which has a wide pocket (556 Å^3) without an entrance. Only the closed model binds to an inhibitor. The two distinct conformations of 15-LOX observed indicate the conformational flexibility of lipoxygenases, which should be considered in corresponding inhibitor design. The structure of the closed conformation may serve as a better template to build a comparative model of 5-LOX for inhibitor design.

In the present study, we have built a comparative model for 5-LOX based on the closed conformation of 15-LOX and used it to screen inhibitors. To consider conformational flexibility, we carried out molecular dynamics simulations to generate possible conformations of 5-LOX binding to a strong inhibitor. The complex structures that provide the most reasonable explanation for the activities of known inhibitors were selected for further virtual screening. A series of novel inhibitors for 5-LOX were identified, and two of them appeared to be potential dual-functional inhibitors of 5-LOX and mPGES-1.

RESULTS

Comparative Modeling of the Human 5-Lipoxygenase Inhibitor Binding Structure. The comparative model of the human 5-LOX closed conformation (Figure 1) was built on the basis of two templates: the revised, closed model structure of rabbit 15-LOX (PDB entry 2P0M, chain B, identity 38.6%)[16] for the whole protein, and the structure of coral 8*R*-LOX (PDB entry 2FNQ, identity 38.8%)[13] for only the N-terminal C2-like domain. The structure of 8*R*-LOX was used because its calcium binding residues were conserved with those of human 5-LOX. The model was evaluated by programs PROCHECK[17] and ProSA-web.[18,19] Both evaluations indicated a good-quality model: for PROCHECK, the overall average *G*-factor was 0.03 (the criteria is larger than −0.5); for PROSA, the *z*-score was −9.89 (673 residues), in the range of native proteins.

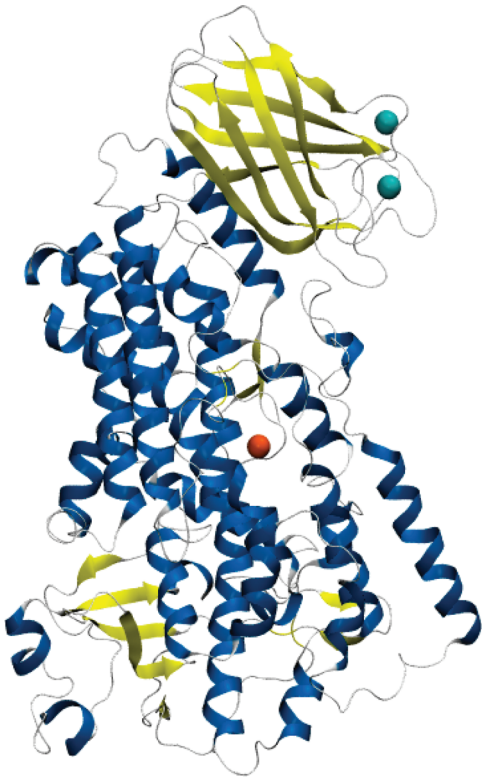


Figure 1. Comparative model of human 5-lipoxygenase in the closed conformation (calcium ion, cyan ball; ferrous ion, red ball).

Refinement of 5-Lipoxygenase−Inhibitor Complex by Molecular Dynamics Simulation. To obtain a better inhibitor binding conformation of 5-LOX, the comparative model was refined on the basis of the interactions with known inhibitors. First, a strong inhibitor (IC_{50} = 10 nM) reported by Hutchinson et al. from Merck-Frosst, (*S*)-2-(2-((1-(3-chlorobenzyl)-4-methyl-6-((5-phenylpyridin-2-yl)methoxy)-4,5-dihydro-1*H*-thiopyrano[2,3,4-*c*,*d*]indol-2-yl)methoxy)phenyl)-acetic acid (**31**, Figure 2),[20] was docked into the substrate

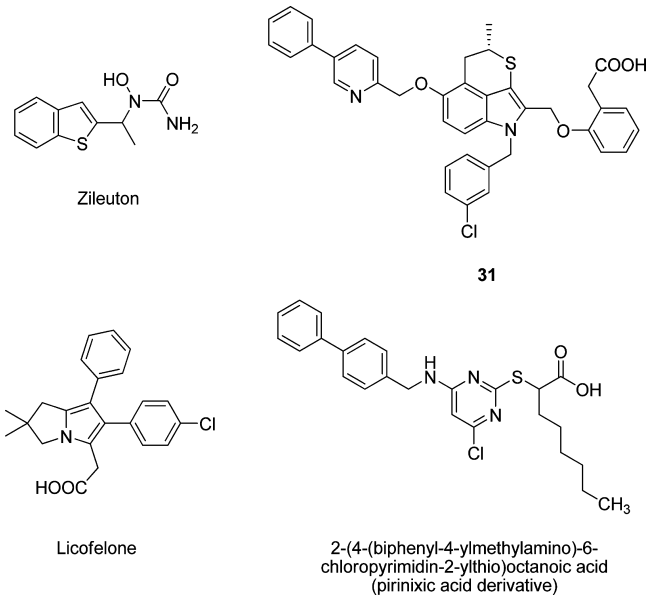


Figure 2. Chemical structure of 5-LOX inhibitors and dual 5-LOX/mPGES-1 inhibitors.

binding site of the comparative model. This compound was selected because it is highly potent and large in size (with a molecular weight of 661.21, which may fill the pocket) and all the chiral centers are specified. Then 20 docked complexes (for the protein, only the lipoxygenase domain, residues 118−673, was used) were submitted to short molecular dynamics (MD) simulations. Conformations of 5-LOX in the trajectories were clustered to 15 clusters with a rms tolerance of 2.0 Å of the substrate binding site residues, and the central structures of each cluster were evaluated by their ability to predict the inhibition activities of known inhibitors. Twenty-five compounds (IC_{50} values ranging from 10 nM to more than 10 μM, reported by Merck-Frosst, Table S1, Supporting Information) were docked into each of the 15 models, and linear regression was done between the pK_i (estimated inhibition constant) and pIC_{50} values. The model with the largest correlation coefficient (R = 0.452) was identified as the best inhibitor binding conformation for 5-LOX and used in the following virtual

dx.doi.org/10.1021/jm201497k | J. Med. Chem. 2012, 55, 2597−2605

screen. This model was also verified using ProSA-web[18,19] with the z-score of −8.67 (556 residues) in the range of native proteins. As a comparison, the structure model before MD refinement was also used to dock the 25 known inhibitors, and the correlation coefficient between the pK_i (estimated inhibition constant) and pIC_{50} values was low (R = 0.149). For details of the refinement procedure, see the Experimental Section.

Virtual Screen. To discover novel inhibitors of 5-LOX, a virtual screen was done using the SPECS compound database (November 2009 version for 10 mg, 201 007 compounds).[21] The 3D structure of the compounds were built with the LigPrep[22] program of Schrödinger software in Maestro 9.0.211 (using default settings). A 3D structure library containing 197 211 compounds was established (generation of 3D structure failed for 3796 compounds). Virtual screening was performed following a three-step scheme combining molecular docking and pharmacophore mapping (Figure 3): (1) all compounds in the library was docked into the inhibitor binding pocket of the refined 5-LOX model using the program DOCK 6.1,[23] and 39 996 compounds were selected (the library was almost equally divided into four groups according to the compound indices, and from each group 9999 top-ranked compound were selected); (2) binding conformations of these 39 996 compounds were evaluated using the pharmacophore-based scoring program PSCORE (an in-house program developed in our laboratory),[24] and 4347 compounds with highest matching scores were selected; (3) these 4347 compounds were docked into the refined 5-LOX model using AutoDock 4.00[25] (flexible ligands and rigid receptor), and the first 1000 compounds with lowest estimated K_i (spanning from 47.79 to 331.38 nM, predicted by AutoDock) were selected. Then binding conformations of these 1000 compounds were exported and evaluated manually according to the following criteria: (1) forming at least two hydrogen bonds, (2) containing at least one ring which has hydrophobic interaction, (3) not being a polypeptide, and (4) containing no metal atoms. In this step, 105 compounds were selected and purchased to test their inhibition to 5-LOX experimentally.

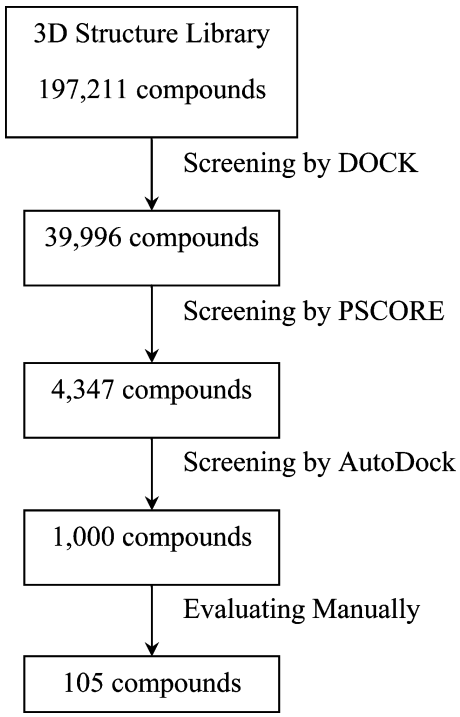


Figure 3. Virtual screen scheme to retrieve inhibitors of human 5-LOX.

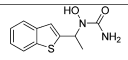
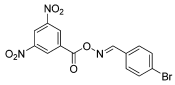
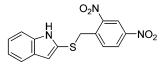
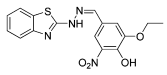
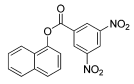
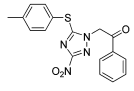
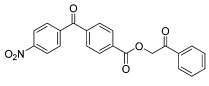
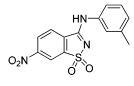
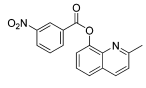
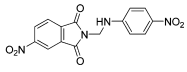
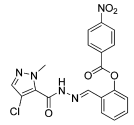
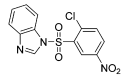
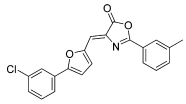
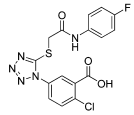
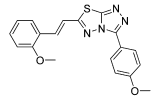
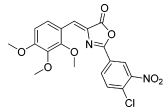
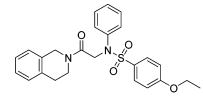
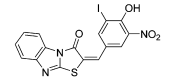
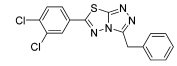
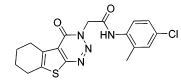
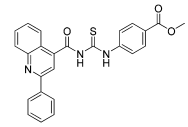
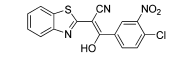
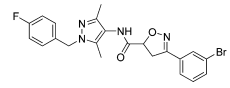
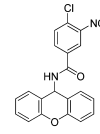
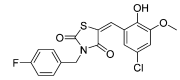
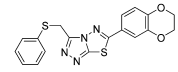
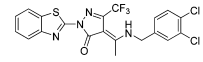
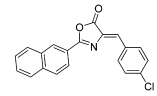
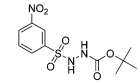
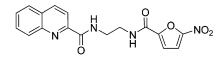
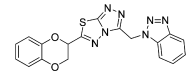
Inhibition Testing. We purchased the 105 selected compounds and tested their inhibition to the activity of recombinant human 5-LOX in a cell-free assay. The purity of compounds from the SPECS database is more than 90% and for most compounds greater than 95% (confirmed by SPECS, using NMR or LC−MS; data available through the Web site). The cell-free assay is based on the oxidation of the substrate 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) to the highly fluorescent product during 5-LOX's catalytic reaction (see Experimental Section).[26] All compounds were soluble in DMSO, and the inhibition values were tested at the concentration of 100 μM. A 5-LOX inhibitor, zileuton, was used as reference control, and DMSO (4.5%, v/v) was used as vehicle control. Thirty of the 105 compounds showed significant inhibition of 5-LOX activity (over 50% inhibition) in the preliminary test (see Table S2 of the Supporting Information for ranks in virtual screening). The IC_{50} values of these 30 compounds were then determined (Table 1; dose−response behavior shown in Figure S1, Supporting Information). Eleven compounds had IC_{50} values less than 10 μM. The inhibition activities of compounds **1**−**4** were even stronger than that of the reference compound zileuton.

The effects of these 30 compounds were also tested in human whole blood stimulated with calcium ionophore A23187, which has been reported to induce the 5-LOX pathway.[27,28] Nine compounds markedly suppressed the production of LTB_4 with IC_{50} values less than 100 μM in the human whole blood assay. Compounds **4**, **7**, and **11** had IC_{50} values less than 10 μM (**4**, 8.6 ± 0.9 μM; **7**, 9.7 ± 1.2 μM; **11**, 8.1 ± 1.5 μM), comparable to that of zileuton (3.3 ± 0.4 μM). The inhibition of compounds **1**−**30** of the 5-LOX downstream enzyme LTA_4H (hydrolase activity) was also tested, and all their inhibition percentages were less than 50% at 100 μM (data not shown), indicating that their intervention of LTB_4 formation was attributable to their inhibition activity on 5-LOX.

As a few 5-LOX inhibitors were also reported to interfere with microsomal prostaglandin E synthase (mPGES)-1,[29] such as licofelone[30] and pirinixic acid derivatives,[31] we also tested the inhibition effects of the identified 5-LOX inhibitors against it. A key enzyme of cyclooxygenases (COX) pathway in the AA cascade, mPGES-1, has attracted much attention as a key drug design target.[32] Compounds **1**−**30** were also evaluated for possible inhibition of the biosynthesis of prostaglandin E_2 (PGE_2), another important inflammatory mediator generated from AA by cyclooxygenases (COX-1 and COX-2) and mPGES-1.[32] Human whole blood stimulated with lipopolysaccharides (LPS), which has been reported to induce COX-2 and mPGES-1,[33,34] was used to investigate the intervention of PGE_2 formation. Inhibition effects to the production of PGE_2 in human whole blood assay by compounds **1**−**30** at 20 μM concentration were measured. Two compounds, **4** and **7**, which were also strong inhibitors of 5-LOX, showed inhibition larger than 50%, with IC_{50} values of 7.6 ± 0.2 and 15.6 ± 0.4 μM, respectively [for the reference control compound 2-(6-chloro-1H-phenanthro[9,10-d]imidazol-2-yl)isophthalonitrile (MF63),[35] IC_{50} = 1.8 ± 0.3 μM] (Table 2). Then the two compounds were tested for their inhibition activity toward recombinant COX-1, COX-2, and mPGES-1 in cell-free assays.

dx.doi.org/10.1021/jm201497k | J. Med. Chem. 2012, 55, 2597−2605

Table 1. Inhibition Activity to Human 5-LOX in Cell-free and Human Whole Blood (HWB) Assay[a]

SPECS ID Number	ID Number in Text	Chemical Structure	IC_{50} (μM) Cell-free	IC_{50} (μM) HWB
-	Zileuton		1.11 ± 0.15	3.7 ± 0.4
AG-690/08564038	1		0.62 ± 0.14	36.0 ± 2.1
AI-204/31730040	2		0.80 ± 0.16	23.9 ± 2.9
AG-690/09625006	3		0.86 ± 0.11	40 ± 5
AE-641/00598013	4		1.04 ± 0.21	8.6 ± 0.9
AP-853/41543842	5		1.4 ± 0.3	76 ± 9
AG-205/11631100	6		1.49 ± 0.15	48 ± 3
AH-034/07126020	7		1.87 ± 0.12	9.7 ± 1.2
AG-670/36678015	8		4.6 ± 0.8	>100
AE-641/05570007	9		5.3 ± 1.8	38 ± 5
AK-968/40388353	10		5.4 ± 1.2	>100
AG-690/11215504	11		6.9 ± 2.1	8.1 ± 1.4
AG-690/37128069	12		14.2 ± 1.0	>100
AN-465/42154556	13		15 ± 3	>100
AO-365/43301118	14		17 ± 2	>100
AK-968/11573163	15		20 ± 4	>100
AO-081/40778633	16		29 ± 4	>100
AG-205/37047027	17		33.8 ± 0.2	>100
AO-365/43113260	18		37.5 ± 1.9	>100
AP-906/42086948	19		44 ± 9	>100
AK-968/41169732	20		45 ± 6	>100
AF-399/36911012	21		53 ± 4	>100
AN-329/43211400	22		65 ± 9	>100
AG-205/08523031	23		67 ± 5	>100
AN-698/42006703	24		68 ± 9	>100
AO-365/43368620	25		70 ± 11	>100
AK-968/12167906	26		70 ± 10	>100
AG-690/37078004	27		80 ± 9	>100
AG-690/15431400	28		81 ± 9	>100
AO-365/43403215	29		82 ± 5	>100
AO-365/43113231	30		97 ± 12	>100

[a]Data are the average values of three separate experiments (standard error of the mean from at least three independent experiments.

Both compounds significantly inhibited the activity of mPGES-1 (IC_{50} values: **4**, 3.6 ± 0.8 μM; **7**, 6.7 ± 0.2 μM; MF63, 3.0 ± 0.1 nM), but they hardly inhibited COX-1 and COX-2 (inhibition lower than 50% at 100 μM), indicating that their intervention of PGE_2 formation is attributable to their inhibition activity on mPGES-1.

DISCUSSION

We have built a model for human 5-LOX in closed conformation and successfully used it in inhibitor virtual screening. The original comparative model was built on the basis of the revised crystal structure of rabbit 15-LOX (PDB entry 2P0M[16]), which includes 15-LOX in a closed

dx.doi.org/10.1021/jm201497k | J. Med. Chem. 2012, 55, 2597−2605

Table 2. Inhibition Activity of Compounds 4 and 7 toward 5-LOX and mPGES-1 in Cell-Free and Human Whole Blood (HWB) Assay, Compared with the Effects of Licofelone[a]

	IC_{50} (μM)			
ID in text	5-LOX (cell-free)	mPGES-1 (cell-free)	LTB_4 (HWB)	PGE_2 (HWB)
zileuton	1.11 ± 0.15		3.3 ± 0.4	
MF63		0.0030 ± 0.0001		1.8 ± 0.3
4	1.04 ± 0.21	3.6 ± 0.8	8.6 ± 0.9	7.6 ± 0.2
7	1.87 ± 0.12	6.7 ± 0.2	9.7 ± 1.2	15.6 ± 0.4
licofelone	0.18[36]	6[37]	3.6[b36]	3.9[36]

[a]Data are the average values of three separate experiments (standard error of the mean from at least three independent experiments. [b]The value was measured in basophilic leukemia cell assay using RBL-1 cell. No IC_{50} values tested in HWB assay have been published.

conformation binding to an inhibitor. Then possible inhibitor binding conformations were generated using MD simulation, and the final model was selected according to its ability to identify known inhibitors. Though the correlation coefficient between the predicted and experimental activities was modest (R = 0.452), it can reasonably explain the interactions of inhibitor **31**, one of the known competitive inhibitors that are too large to be docked into the open conformation of 5-LOX. In the refined model, **31** forms hydrogen bonds to Gln363, Thr364, Lys409, Gln413, and the main chain nitrogen atom of Leu414 and has hydrophobic interactions (Figure 4).

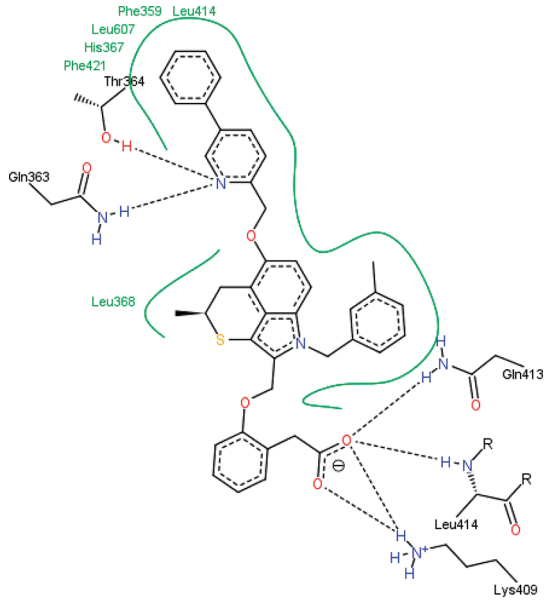


Figure 4. Interaction between **31** and 5-LOX (generated by software PoseView[40]).

Furthermore, virtual screening using this model successfully identified 5-LOX inhibitors. The success rate of about 29% (30 out of 105 virtual screened compounds) validated the model and its effectiveness in virtual screening. As there is no complexed strucuture of 5-LOX available and the protein is quite flexible upon ligand binding (e.g., in the case of 15-LOX), our model gives one possible conformation for 5-LOX binding to large inhibitors. The protein may be freezed to distinct conformations when binding to different inhibitors, which might be one of the possible reasons for the modest correlation coefficient between the predicted and experimental activities of the 25 known inhibitors when docked to the model that is best fit to **31**. When the binding conformation of the target protein was unknown, ligand-based approaches, such as pharmacophore, QSAR, or pseudoreceptor method, can be used for inhibitor screen. As there is no ligand binding structures of 5-LOX available, Hofmann et al. used ligand-based virtual screening to identify new inhibitors of 5-LOX with activities at the micromole range.[38] They recently used pseudoreceptor models to screen for potent inhibitors of 5-LOX and successfully identified a new inhibitor with micromole IC_{50} using intact polymorphonuclear leukocytes.[39] Though we searched the same SPECS database, none of our active compounds share similar chemcial structures with those discovered by Hofmann et al., indicating the complementarity of the ligand-based and protein-based approaches.

In January 2011, the crystal structure of human 5-LOX was reported.[11] This is a monumental work in the field of 5-LOX, which provides important information on the inhibitor binding site. However, this crystal structure only gives an apo-structure without any inhibitor binding. The volume of the pocket is 255 Å^3, much smaller than that of the virtual screening model, 922 Å^3 (pockets were detected using the program Cavity 1.0[41]). We also tried to use the newly reported crystal structure to dock the known inhibitors (the same method to evaluate the models generated by MD simulation) and to see whether a good predictive model can be got. Unfortunately, the correlation coefficient between the predicted and experimental IC_{50} was negative (−0.224). Thus, this crystal structure may not be used directly for inhibitor screen before large-scale structure flexibility analysis is carried out. In our refined model, the residues 603−614 move outside, making the cavity larger. We also docked our screened compounds (**1**−**30**) into the pocket of the crystal structure (100 separate docking simulations were performed for each compound, using AutoDock), and obtained no correlation between the predicted binding constants and the log IC_{50} values (R = 0.091), while the correlation coefficient is much higher using our structure model (R = 0.319).

The active compounds we identified are all novel 5-LOX inhibitors with unique chemical structures. None of the compounds **1**−**30** has been reported as 5-LOX inhibitor or shares the same scaffold with known inhibitors. For the three compounds **4**, **7**, and **11** with IC_{50} values less than 10 μM in human whole blood assay, we searched in SciFinder for substances with similarity larger than 80% to the three compounds, and none of the references were related to inflammatory study. These compounds may be developed into new anti-inflammatory drugs or provide new scaffolds to develop 5-LOX inhibitors.

Compounds **4** and **7** were found to be dual-functional inhibitors of 5-LOX/mPGES-1. AA can also be metabolized to prostaglandins, another class of inflammatory mediators, through the COX pathway.[42] COX-1 and COX-2 convert AA to prostaglandin H_2 (PGH_2), and PGH_2 is subsequently

dx.doi.org/10.1021/jm201497k | J. Med. Chem. 2012, 55, 2597−2605

metabolized to prostanoids by several terminal prostanoid synthases.[43] mPGES-1, which catalyzes the conversion of PGH_2 to PGE_2, has been proposed as a potential target for therapeutic intervention in inflammatory-related diseases.[44] It has been shown that interfering in both the 5-LOX pathway and COX pathway is an effective strategy in treating inflammation, and multifunctional inhibitors to both pathways are highly sought.[45−47] Moreover, targeting 5-LOX and mPGES-1 could selectively suppress PGE_2 and may have less toxicity than dual 5-LOX/COX-2 inhibitors. In fact, licofelone (ML3000), a drug against osteoarthritis in phase III clinical trial, is an inhibitor of 5-LOX, COX-1 rather than COX-2, and mPGES-1.[48,49] We have discovered that compounds **4** and **7** simultaneously suppressed the production of LTB_4 and PGE_2 in human whole blood, and their targets were verified as 5-LOX and mPGES-1, not LTA_4H, COX-1, COX-2. The IC_{50} values were in micromolar range and comparable to the known dual-functional inhibitors of 5-LOX/mPGES-1 (such as licofelone, arylpyrrolizines,[50] and pirinixic acid derivatives[31]). Compound **4** was an intermediate for the preparation of polyimides.[51] The application of **7** was not reported. The two compounds provide new scaffolds to design multifunctional inhibitors to enzymes in AA metabolic network.

To conclude, we have built a comparative model for the human 5-LOX closed conformation and successfully used it in virtual screening. Out of about 200 000 compounds in the SPECS database, 105 compounds were selected for experimental test. In cell-free assay, 30 compounds were found to have IC_{50} values less than 100 μM and 11 with IC_{50} values less than 10 μM. Compounds **4**, **7**, and **11** had inhibition activity in the human whole blood assay with IC_{50} values less than 10 μM. Compounds **4** and **7** were also discovered as efficient dual-functional inhibitors of 5-LOX and mPGES-1 in both cell-free assay and cell-based assay. The interference with several anti-inflammatory targets from the arachidonic acid cascade should provide benefits in pharmacotherapy in terms of synergistic therapeutic effects as well as reduction of the incidence of typical NSAID-related side effects. These novel lead structures may be regarded as potential candidates for anti-inflammation treatment.

The success of using the comparative model of human 5-LOX with closed conformation in virtual screening indicates that conformational flexibility should be considered while designing inhibitors against 5-LOX. We sampled the 5-LOX flexibility by docking known inhibitors into the pocket followed by molecular dynamics simulations. The final model was chosen according to the ability to predict the activities of known inhibitors. This strategy may be applied to the newly reported crystal structure of human 5-LOX to build a more accurate model of 5-LOX in inhibitor binding structure.

EXPERIMENTAL SECTION

Comparative Modeling. Comparative modeling was performed using the program MODELER 9v6[52] in three steps: (1) superpose the structures of closed form rabbit 15-LOX (2P0M, chain B) and coral 8*R*-LOX (2FNQ) and generate an alignment of their sequences (using the script salign.py), (2) align the sequence of human 5-LOX to the alignment of the templates (align2d_mult.py), and (3) generate five models of human 5-LOX binding to one ferrous ion and two calcium ions (model-ligand.py). All five models were geometry-optimized using GROMACS 4.0.4[53] following this scheme: (1) geometry-optimize the structures in OPLS-AA/L all-atom force field, fixing the position of backbone atoms, and (2) remove the ferrous ion at the catalytic site and geometry-optimize the structures in GROMOS96 43a1 force field, fixing the position of atoms binding to the ferrous (His367 Nε, His372 Nε, His550 Nε, Asn554 Oδ, Ile673 terminal carboxyl oxygens). From the five geometry-optimized models, the one with the largest substrate binding pocket was selected for further study.

Molecular Docking. To dock known inhibitors into 5-LOX models, molecular docking with flexible ligands and rigid receptor was performed with the program AutoDock 4.00.[25] Lamarckian genetic algorithm was used with following parameters: number of individuals in population, 300; maximum number of energy evaluations, 25 000 000; maximum of generations, 27 000. Docking results were clustered with a rms tolerance of 2.0 Å.

For docking compound **31** in the comparative model, 100 molecular docking simulations were performed. The lowest energy conformations from each of the first 20 clusters (estimated K_i between 2.86 and 267.68 mM) were taken as possible binding modes and used for further study. For docking the 25 compounds into the 15 models generated by MD simulation, 20 molecular docking simulations were performed. The lowest estimated K_i of a compound in the 15 models was used for linear regression.

For the DOCK step in virtual screening, the rigid-body docking approach was used with default parameters.

For the AutoDock step in virtual screening, the Lamarckian genetic algorithm was used with the following parameters: number of genetic algorithm runs, 20; number of individuals in population, 150; maximum number of energy evaluations, 5 000 000; maximum of generations, 27 000. The first 1000 compounds with lowest estimated K_i (spanning from 47.79 to 333.42 nM) were selected.

Molecular Dynamics Simulation. The MD simulations were run with GROMACS 4.0.4 using GROMOS96 43a1 force field. The topology files of small molecules were generated using the Dundee PRODRG2.5 server (beta) (davapc1.bioch.dndee.ac.uk/cgi-bin/prodrg_beta). Each complex structure was geometry-optimized and then put into a 82 × 73 × 79 Å^3 water box containing 11 702−11 713 (depending on the complex structures) water molecules described by SPC model[54] and 12 sodium ions as counterions. Each system was geometry-optimized, balanced with position-restrained MD for 1 ns (total energy was stable), and run without restraints for 5 ns. Long-range electrostatic interactions were calculated using PME methods. Temperature was kept at 350 K using the v-rescale method, and pressure was coupled to 1.0 atm using the Parrinello−Rahman method. The snapshot structures were saved for every 5 ps. Of all the 20 MD simulations, 18 successfully completed, but two failed due the structure collapse around the inhibitor binding pocket, which were discarded. Conformations between 1 to 5 ns of the 18 trajectories were clustered using the g_cluster command. The linkage method was used, and the rmsd cutoff of the residues at the substrate binding pocket (40 residues: 147, 169, 175−178, 180, 359, 360, 362−365, 367, 368, 372, 405−407, 409−411, 413, 414, 420, 421, 423−425, 427, 554, 557, 569, 600, 602−604, 606, 607, 673) was set to 2.0 Å. A total of 14 418 conformations were classified into 15 clusters. The central structures of each cluster were geometry-optimized by energy minimization and taken as candidates for the best inhibitor binding model.

Molecular Cloning and Protein Expression of 5-LOX. The cDNA sequence of human 5-LOX was subcloned into the pET-21 system from pT3−5LOX, which was kindly donated by Prof. Olof Radmark (Department of Medical Biochemistry and Biophysics, Division of Physiological Chemistry II, Karolinska Institute, Sweden).[55] The 5-LOX coding region was ligated into the pET21a(+) vector followed by DNA sequencing confirmation (Invitrogen) and transformed to the BL21-DE3 strain of *Escherichia coli*. For expression, the cell culture was cultivated at 37 °C until the OD_{600} reached 0.4−0.6. Then 5-LOX expression was induced, and the cells were grown for another 16−20 h at 18 °C. The cells were harvested by centrifugation at 6000 rpm for 10 min at 4 °C. The cell pellets were resuspended in solubilizing buffer (50 mM Tris/HCl, pH 7.3, 0.1 mM EDTA, 2 mM dithiothreitol) with 1 mM phenylmethanesulfonyl fluoride and 0.5 mg/mL lysozyme. Cell lysis was obtained by ultrasonication and followed by centrifugation at 17 000 rpm for 25 min at 4 °C. The supernatant was aliquoted and frozen at −80 °C until use.

Molecular Cloning and Protein Expression of mPGES-1. The cDNA sequence of mPGES-1 was subcloned into pET28a(+) vector and transformed to the Rosetta-DE3 strain of *E. coli* for protein expression. Protein expression was induced when OD_{600} reached 1.0 and continued growing at 25 °C for 12 h. The cells were harvested by centrifugation at 5000*g* for 20 min at 4 °C and broken by sonication. Insoluble material was separated by centrifugation at 12 000 g for 30 min at 4 °C. The supernatant was then ultracentrifuged at 174 000*g* for 1 h at 4 °C. The membrane pellet was washed and resuspended in solubilizing buffer. Protein concentrations were measured using a bicinchoninic acid protein assay kit (Biomed).

Inhibition Assay of 5-LOX in Cell-Free Systems. The enzyme activity of 5-LOX was determined fluorescence spectrophotometrically by oxidation of the substrate H_2DCFDA to the highly fluorescent 2′,7′-dichlorofluorescein product during 5-LOX's catalytic reaction.[26] The enzyme was incubated at room temperature in 96-well microtiter plates (Costar, Corning Inc.) in 50 mM Tris-HCl, 0.2 mM ATP, 0.1 mM dithiothreitol, 0.1 mM EDTA, 0.5 mM $CaCl_2$, and 10 μM H_2DCFDA (pH 7.5). After 10 min of preincubation of inhibitor (dissolved in DMSO) and enzyme, the reactions were initiated by the addition of arachidonic acid as substrate and then monitored by excitation at 500 nm and emission at 520 nm utilizing a multiwell fluorometer (Synergy4, BIOTEK). Fluorescence signals were recorded for 5 min with a kinetics mode program. For the IC_{50} determinations, 25 μM arachidonic acid and 2.5% final DMSO concentration were used. The initial reaction rates at different inhibitor concentrations were used for IC_{50} determinations, which were calculated using a four-parameter logistical model of the graph of log dose against percentage inhibition and were obtained from at least three sets of experiments.

Inhibition Assay of mPGES-1 in Cell-Free Systems. The enzyme activity of mPGES-1 was determined by assessment of PGH_2 conversion to PGE_2.[56] Briefly, PGH_2 was added to each well of a 96-well plate and the reaction was started by the addition of a microsome sample. The reaction was terminated by adding stop solution after reacting at 4 °C for 1 min. The PGE_2 production in the reaction mixture was determined using the PGE_2 EIA kit (Cayman Chemical). For the IC_{50} determinations, 2.5 mM cofactor and 17 μM PGH_2 were used.[57,58] Enzyme samples were preincubated with inhibitor for 15 min at 4 °C.

Inhibition Assay of COX-2 and COX-1 in Cell-Free Systems. The enzyme activities of COXs were determined spectrophotometrically by oxidation of TMPD during the conversion of PGG_2 to PGH_2.[59] Inhibition activities were measured as described.[60]

Inhibition Assay of LTA_4H in Cell-Free Systems. The LTA_4H hydrolase activity was measured by the formation of LTB_4 using an ELISA assay. Inhibition activities were measured as described.[61]

Measuring PGE_2 and LTB_4 Formation in Human Whole Blood. Fresh human blood was obtained from healthy volunteers, who did not received NSAIDs for at least 14 days. Coagulation was prevented with heparin (J&K Chemical LTD) at 10 UI/mL, and 0.5 mL of blood was immediately aliquoted into a series of PP tubes. Blood samples were preincubated with either vehicle (DMSO) or various concentrations of test compounds DMSO solutions for 20 min at 37 °C. To assay LTB_4 formation, calcium ionophore A23187 (Sigma) was added to the blood with the final concentration of 20 μg/mL, followed by incubation for 0.5 h at 37 °C to induce LTB_4 formation. To assay PGE_2 formation, LPS (Sigma) was added to the blood with the final concentration of 100 μg/mL, followed by incubation for 24 h at 37 °C to induce PGE_2 formation. The incubation was terminated by centrifugation for 5 min at 3000*g* at 4 °C to obtain plasma. The plasma was frozen at −80 °C until use. To determinate eicosanoid production, LTB_4 and PGE_2 EIA kits (Cayman Chemical) were used according to the manufacturer's instructions.

■ ASSOCIATED CONTENT

Supporting Information

Chemical structure and reported inhibition activities of known inhibitors used to evaluate the candidate protein structure models, rank in virtual screening of compounds active in cell-free assay, and dose−response behavior of screened inhibitors. 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.

■ ACKNOWLEDGMENTS

This research was supported by the Ministry of Science and Technology of China (2009CB918500, 2009ZX09501) and the National Natural Science Foundation of China (20873003, 11021463).

■ ABBREVIATIONS USED

AA, arachidonic acid; COX, cyclooxygenase; 5-LOX, 5-lipoxygenase; HWB, human whole blood; LTA_4H, leukotriene A_4 hydrolase; LTB_4, leukotriene B_4; mPGES-1, microsomal prostaglandin E synthase-1; PGE_2, prostaglandin E_2; PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; PLA_2, phospholipase A_2

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