Discovery of Multitarget Inhibitors by Combining Molecular Docking with Common Pharmacophore Matching

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Multitarget drugs have been to be found effective in controlling complex diseases. However, how to design multitarget drugs presents a great challenge. We have developed a computer-assisted strategy to screen for multitarget inhibitors using a combination of molecular docking and common pharmacophore matching. This strategy was successfully applied to screen for dual-target inhibitors against both the human leukotriene A_4 hydrolase (LTA4H-h) and the human nonpancreatic secretory phospholipase A2 (hnps-PLA2). Three compounds screened from the chemical database MDL Available Chemical Directory were found to inhibit these two enzymes at the 10 μM level. Moreover, one synthetic compound matching the common pharmacophores inhibits LTA4H-h and hnps-PLA2 with IC_{50} values of 35 nM and 7.3 μM, respectively. The common pharmacophore model can also be used to search small molecule databases or collections of existing inhibitors, as well as to guide combinatorial library design to search for ideal multitarget inhibitors.

Introduction

With the emergence of in vitro biochemical assays and the development of biochemical and genetic studies, the drug discovery paradigm has shifted from the animal model screening approach to the target-focused ligand discovery model.[1] This concept has led to many drugs reaching the market; however, the number of successful drugs and novel targets has fallen significantly behind expectations in recent decades.[2−4] A possible reason for this is the redundancy and robustness in many biological networks and pathways in which cells often find ways to compensate for a protein whose activity is affected by a drug. Modulating multiple targets in the biological network simultaneously is recognized to be beneficial for treating a range of diseases, such as acquired immune deficiency syndrome (AIDS), atherosclerosis, cancer, and depression, and this recognition has led to a growing trend to design multiple-target drugs.

Combinations of drugs, a form of multitargeting, combining different inhibitors that target specific single target, are the standard treatment for diseases, including cancer, type 2 diabetes mellitus, and viral and bacterial infections.[5−7] A new generation of multitarget drugs, single chemical entity that act on multiple molecular targets, is emerging; examples are (4*S*,7*S*,10a*S*)-5-oxo-4-{[(2*S*)-3-phenyl-2-sulfanylpropanoyl]amino}-2,3,4,7,8,9,10,10a-octahydropyrido[6,1-*b*][1,3]thiazepine-7-carboxylic acid (omapatrilat) (a dual angiotensin-converting enzyme and neutral endopeptidase inhibitor) and 5-((6-((2-fluorophenyl)methoxy)-2-naphthalenyl) methyl)-2,4-thiazolidinedione (netoglitazone) (a peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ agonist).[1] Compared with combinations of drugs, multitarget ligands have more predictable pharmacokinetic and pharmacodynamic relationships as a consequence of the administration of a single drug.[1]

Many multitarget drugs are in clinical use today, but the discovery process is serendipitous, and their modes of action are usually elucidated retrospectively.[1] Designing multitarget inhibitors with predefined biological profiles presents a new challenge. Integrating structural elements from multiple single-target ligands is a predominant technique used for the generation of designed multitarget ligands,[1,8] but many of the reported examples are inefficient in terms of their binding energy per unit of molecular weight because the integrated ligands contain groups that are only important for one of the targets and are merely tolerated by the other targets.[9,10]

Hopkins and his co-workers have shown a correlation between the mean molecular weight of a compound and the total number of targets that this compound shows at least minimal activity against (IC_{50} < 10 μM). This suggests that multitarget compounds tend to be smaller than highly target-selective ones.[9,11] In view of the low molecular weights of multitarget ligands and the efficiency in terms of their binding energy per heavy atom, Morphy and Rankovic have proposed a fragment-based approach: screening a library of fragments to find inhibitors that bind with multiple targets.[9] Although it is possible to obtain some hits through screening, the significant workload and cost to experimentally test all fragments against each target and the low hit rates delivering suitable combinations of activities make this approach difficult to use. The above two approaches to design multitarget ligands have ignored an important source, protein structural information, which can be obtained more conveniently with advances in experimental techniques. In view of this, we propose a novel strategy (Figure 1) that combines protein-based common pharmacophores with molecular docking of small molecules to discover multitarget inhibitors.

To gain more insight into anti-inflammatory drug design, in a previous study, we constructed a computational model for the arachidonic acid metabolic network in human polymorphonuclear leukocytes and studied the dynamic properties of the network.[12] The results showed that flux balance in the network is important for efficient and safe drug design and that the multitarget inhibitors are more effective over a large concentra-

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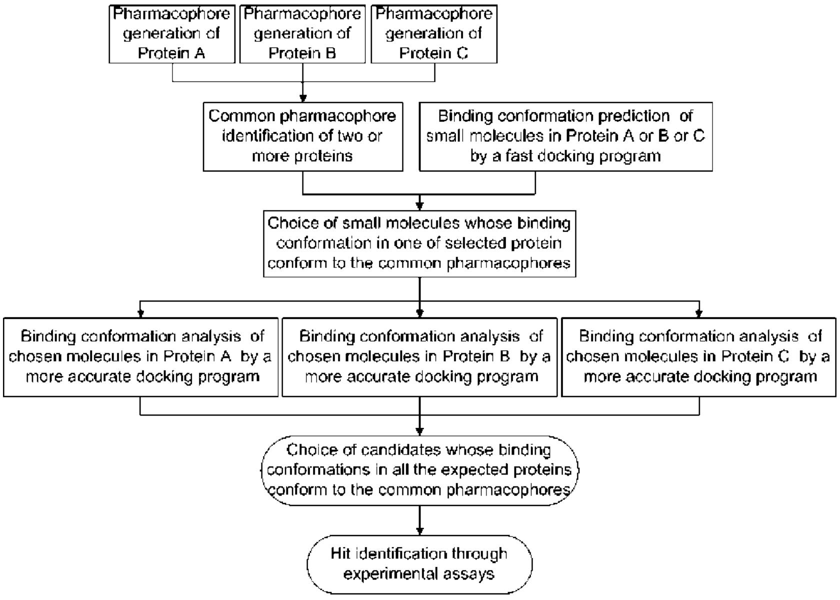

Figure 1. Strategy of multitarget inhibitor discovery.

tion range and are more robust against concentration fluctuations than single-target inhibitors. In the arachidonic acid metabolic network, phospholipase A2 (PLA2) cleaves membrane phospholipids to release arachidonic acid, which is the precursor to a large family of proinflammatory eicosanoids, including prostaglandins and leukotrienes that have been shown to exacerbate many diseases that have an inflammatory component.[13] Inhibition of PLA2 depletes the sources of arachidonic acid and controls the generation of downstream metabolites that are not beneficial to anti-inflammatory processes. On the other hand, leukotriene B_4 (5(*S*),12(*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid, LTB_4[a]), one of the leukotrienes, plays a significant role in the amplification of many inflammatory disease states, and it would be useful to inhibit its biosynthesis. LTB_4 is generated by the hydrolysis of the epoxide LTA_4 (5(*S*)-*trans*-5,6-oxido-7, 9-*trans*-11,14-*cis*-eicosatertraenoic acid) with catalysis by the human leukotriene A_4 hydrolase (LTA4H-h). Therefore, inhibition of LTA4H-h is also a particularly attractive therapeutic approach.[14] Thus, pharmacologically active compounds that simultaneously inhibit PLA2 and LTA4H-h have potential to provide relief for patients suffering from inflammatory disease. In this report, we demonstrate how to discover compounds that inhibit PLA2 and LTA4H-h simultaneously by using our strategy of multitarget inhibitor design based on common pharmacophores.

Results

Multitarget Inhibitor Design Strategy. The concept of the pharmacophore, a set of molecular structural features that is recognized at the binding site and responsible for the biological activity of molecules, is widely used in medicinal chemistry. Pharmacophore models are usually generated from a set of the known active ligands of a target receptor. Deriving a pharmacophore model from the three-dimensional structure of a target protein is an alternative approach that reveals the key elements of the protein−ligand binding site more directly. Our strategy (Figure 1) to discover multiple target inhibitors starts from protein-based pharmacophores by

(1) generating pharmacophore models for each protein under study using Pocket v.2,[15] a program developed in our laboratory to derive pharmacophore models based on protein structures without human intervention;

(2) identifying the common pharmacophores by comparing the pharmacophore models of multiple proteins, if the common pharmacophores exist;

(3) predicting the binding conformations of small molecules of databases in one of the target proteins by a fast docking approach and then selecting those molecules whose binding conformations accommodate the common pharmacophores. This procedure was aided by Pscore, an in-house program that was developed in our laboratory to check whether the binding conformations of compounds in the binding pocket accommodate the designated pharmacophores;

(4) analyzing the binding conformations of the selected compounds in multiple target proteins using a more rigorous docking approach and choosing the candidates whose binding conformations in all the expected proteins accommodate the common pharmacophores by Pscore and human experience;

(5) experimentally testing whether these candidates can indeed inhibit the activities of these target proteins.

Pharmacophore models for LTA4H-h and Human Nonpancreatic Secretory Phospholipase A2 (hnps-PLA2). The complex structure (PDB code 1hs6) of LTA4H-h with 2-(3-amino-2-hydroxy- 4-phenylbutyrylamino)-4-methyl-pentanoic acid (bestatin)[16] and the structure (PDB code 1db4) of human nonpancreatic secretory phospholipase A2 (hnps-PLA2) with [3-(1-benzyl-3-carbamoylmethyl-2-methyl-1*H*-indol-5-yloxy)propyl]phosphonic acid (indole **8**)[17] were used to derive pharmacophores for these two proteins by Pocket v.2. For LTA4H-h, three hydrophobic pharmacophores (three cyan balls at the bottom of Figure 2a) were derived from the narrow, deep L-shaped hydrophobic pocket, and residues Trp-311, Leu-369, The-314, Pro-374, Tyr-267, and Tyr-378 contributed greatly to the hydrophobic interactions. The coordination with metal zinc formed the next pharmacophore (yellow ball in Figure 2a). Another hydrophobic center (cyan ball at the top of Figure 2a) was formed by the residues Val-292 and Tyr-383. The other important pharmacophore was the H-bond acceptor center (red

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[a] Abbreviations: LTA4H-h, leukotriene A_4 hydrolase; hnps-PLA2, human nonpancreatic secretory phospholipase A2; LTA_4, leukotriene A_4; LTB_4, leukotriene B_4.

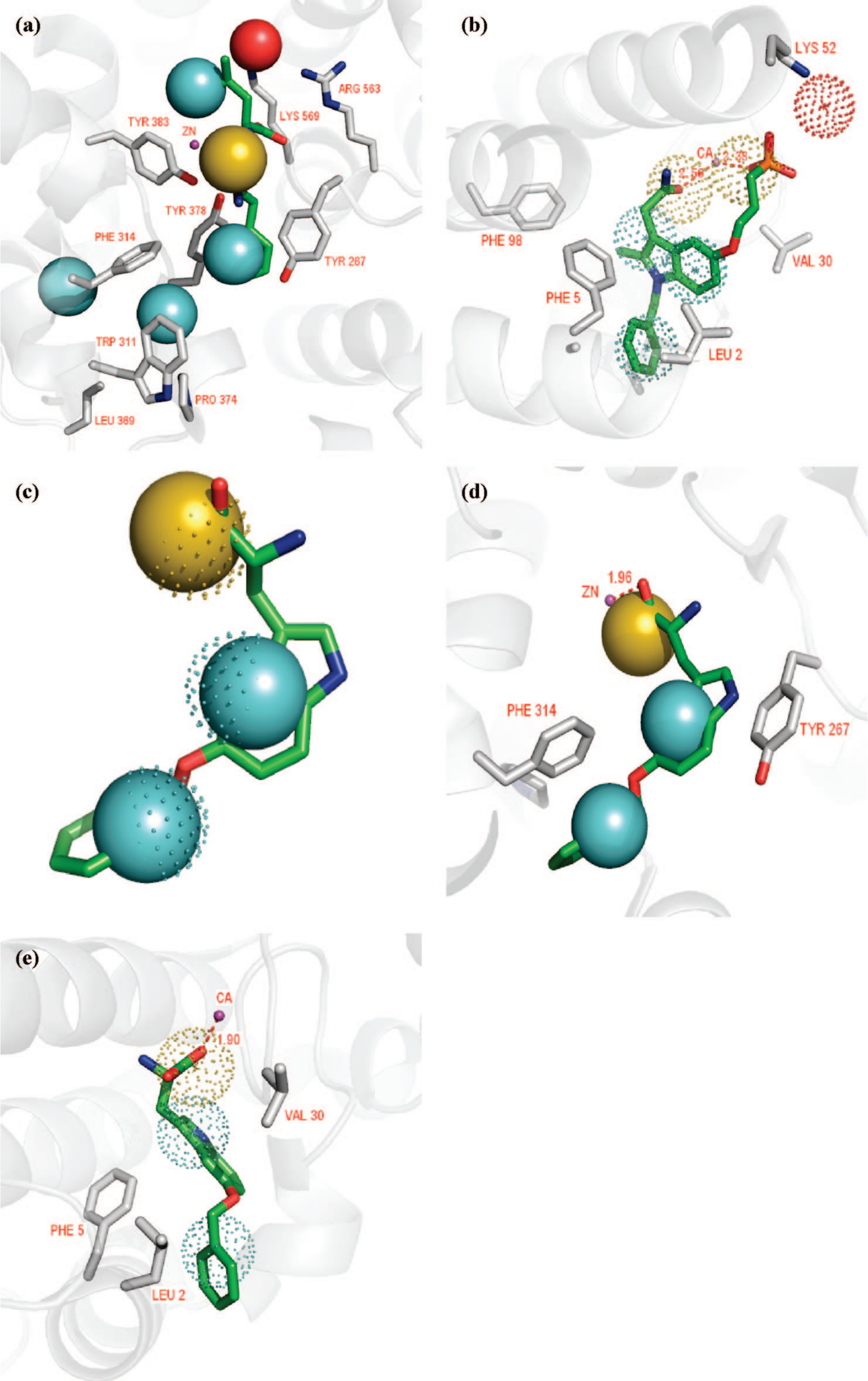
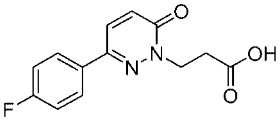
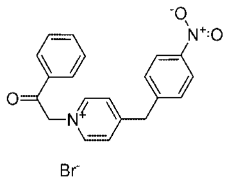
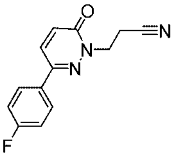
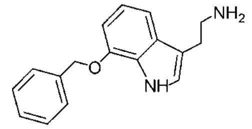
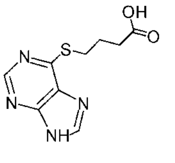
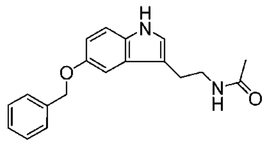
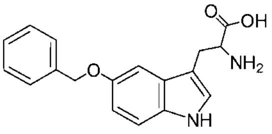
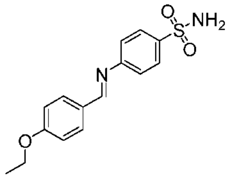
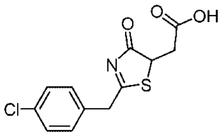


Figure 2. Pharmacophores and inhibitors of LTA4H-h and hnps-PLA2. Solid spheres represent the pharmacophores of LTA4H-h, and dotted spheres represent the pharmacophores of hnps-PLA2. Cyan spheres stand for hydrophobic centers; red spheres represent the H-bond acceptor center; yellow spheres stand for the feature that coordinates with a metal. To clearly show the interactions between the metals and other atoms, the radii of metals are not shown in realistic ratios. (a) Pharmacophore model of LTA4H-h; the inhibitor (bestain) is shown. (b) Pharmacophore model of hnps-PLA2; the inhibitor (indole 8) is shown. (c) Alignment of common pharmacophores of LTA4H-h and hnps-PLA2; the inhibitor compound **4** is shown. (d) Interaction model of compound **4** with LTA4H-h. (e) Interaction model of compound **4** with hnps-PLA2.

ball in Figure 2a) which showed H-bond interaction with Arg-563 and Lys-565.

In the binding pocket of hnps-PLA2, residues Leu-2, Ala-18, Ala-17, Val-30, Phe-5, Phe-98, and Phe-23 formed three hydrophobic centers (three cyan dotted balls in Figure 2b), and two pharmacophores (yellow dotted balls in Figure 2b) were derived from the coordination with a calcium ion. Another H-bond acceptor center (red dotted ball in Figure 2b) was derived from the H-bond interaction between the small molecule and residue Lys-52. Comparing these two sets of pharmacophores (parts a and b of Figure 2), we found that two hydrophobic pharmacophores and a pharmacophore that coordinated with a

Table 1. Nine Candidate Compounds Commercially Available

Compound	Structure	Compound	Structure
1		**6**	
2		**7**	
3		**8**	
4		**9**	
5			

metal (Figure 2c) were common to both proteins. Compounds whose binding conformations in the proteins accommodate the common pharmacophores were expected to inhibit both proteins simultaneously.

Virtual Screening for Compounds That May Bind to Both LTA4H-h and hnps-PLA2. The binding conformations of compounds (in the chemical database MDL Available Chemical Directory) with LTA4H-h were predicted by using Dock 4.0 on the Linux Cluster Platform. For compounds that might bind with both proteins at the same time, high affinity was not expected because a small molecule would be unlikely to bind to a number of different targets with equally high affinity.[5] Compounds with low predicted scores were also likely to be hits, so the binding conformations of the top 150 000 compounds (60% of the database) ranked by docking energy-score in Dock 4.0 were extracted and were checked to determine whether their binding conformations accommodated the common pharmacophores. After this step, 163 compounds (Table S2, Supporting Information) were selected. The binding conformations of these compounds with LTA4H-h and hnps-PLA2 were further analyzed using Autodock 3.05, a program that predicts binding conformations more accurately but takes more time. After a check of whether the binding conformations predicted by Autodock 3.05 matched the common pharmacophores and whether other interactions contributed to the binding, nine compounds (Table 1) were purchased from Sigma-Aldrich, Inc., and J&K Chemical Ltd. The binding conformations of these nine compounds matched well with the common pharmacophores. For example, in the binding conformations of compound **4** with LTA4H-h (Figure 2d) and hnps-PLA2 (Figure 2e), the phenyl ring and the indole ring matched the two hydrophobic centers, and the carboxyl group coordinated with the metals (matching the yellow ball in the common pharmacophores).

Activities against LTA4H-h and hnps-PLA2. LTA4H-h is a 69 kDa zinc-containing enzyme that stereospecifically catalyzes the hydrolysis of the unstable epoxide LTA_4 to the diol LTB_4. The enzyme possesses both intrinsic aminopeptidase activity and epoxide hydrolase activity toward different substrates.[18] The substrate binding pockets of these two activities overlap largely, and both activities are used to measure the inhibition of compounds against LTA4H-h. The inhibition of selected compounds against LTA4H-h and hnps-PLA_2 was measured on the basis of reported methods.[19−21] Among the nine compounds tested, three (corresponding MDL numbers unique to the compounds in chemical database MDL Available Chemical Directory were shown in Table S1 in the Supporting Information) showed activity against the aminopeptidase activity and the epoxide hydrolase activity of LTA4H-h and the activity of hnps-PLA2 (data shown in Table 2, IC_{50} curves shown in Figure S2−S5 in the Supporting Information).

Discussion

Phenotypic observations from large-scale gene deletion studies in several model organisms have shown that biological systems are remarkably resilient to attack and perturbation.[22,23] To rationalize this robustness and redundancy, network analyses have been applied to biological systems and have indicated that

Table 2. IC_{50} Values of Active Compounds against LTA4H-h and hnps-PLA2[a]

	IC_{50} (μM)		
	LTA4H-h		
compd	aminopeptidase	epoxide hydrolase	hnps-PLA2
4	17.3 ± 0.2	231 ± 4	42.1 ± 1.9
6	23.4 ± 2.3	386 ± 5	43.7 ± 1.2
7	12.5 ± 1.3	217 ± 4	52.5 ± 9.0
11	0.228 ± 0.014	0.035 ± 0.002	7.3 ± 1.0

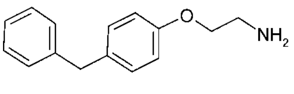
[a] Data are the average values of three separate experiments ± standard error of the mean from at least three independent experiments.

partial inhibition of several targets can be more efficient than complete inhibition of a single target.[11,12] Clinical success of multiple-target drugs in some cases further reveals that improved treatment of complex diseases like cancer should be aimed at several related targets. Increasing attention is being drawn to the design of multitarget inhibitors, but this presents great challenges. In the current study, we proposed a novel and convenient strategy to screen for multitarget inhibitors by checking whether the predicted molecular binding conformations accommodated the target-based common pharmacophores that were identified by comparing the pharmacophores derived from multiple proteins.

Advantage of Choosing Hits under the Restriction of Common Pharmacophores. The great advantage of using this common pharmacophore strategy lies in its ability to reduce potentially astronomical numbers of potential candidates to a more treatable number. Generally, because of the huge number of small molecules put into virtual screening, only the top 10% or less, ranked by docked energy-score, were considered candidates for later evaluation. On the basis of the docking scores for all the compounds from the Available Chemical Directory (248 000 compounds), in the virtual screen of LTA4H-h, the orders of the three active compounds obtained were 5368th, 42 294th, and 140 243rd, and in the virtual screen of hnps-PLA2 (process not shown), the orders were 6272nd, 88 291st, and 25 928th. By use of the top 10% rule, only one compound (compound **4**) would be considered after the first-round virtual screening. In view of the low affinity of multitarget inhibitors, it is necessary to check compounds with low scores, but the workload is large. Under the restriction of the common pharmacophores, the conformations of 150 000 compounds from ACD (60% of the database) were checked, and the number of candidates that needed analysis was further reduced to 163, making it feasible to analyze with a more accurate but more time-consuming docking program. In this way, the common pharmacophore strategy makes it more feasible to screen for low-affinity multitarget inhibitors by computer-assisted virtual screening.

Low Affinity Does Not Mean Low Efficiency. Multitarget inhibitors are thought to have low affinities, but this does not necessarily mean low efficiency at the system level; on the contrary, this strategy has many beneficial effects, such as avoiding drug resistance and toxicity, stabilizing "sick" cells.[24] Compared with single-target inhibitors, the affinity of the three compounds was low, but for a multitarget inhibitor, they may be sufficient and may lead to superior therapeutic effects and side-effect profiles. For example, 1-amino-3,5-dimethyl-tricyclo[3,3,1^{3,7}]decane (memantine) (used to treat Alzheimer's disease) and other multitarget noncompetitive *N*-methyl-D-aspartate receptor antagonists only weakly inhibit multiple targets, [25−28] but they are used because of the reduced range of side effects, compared with high-affinity single-target inhibitors.

Chart 1. Compound **10**, a Reported Inhibitor of LTA4H-h, and Compound **11**, a Synthetic Analogue of Compound **10**



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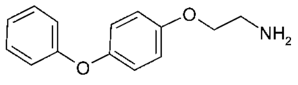

11

Of course, further in vivo studies are necessary to investigate the overall effects of these three compounds at the system level.

Further Applications of the Common Pharmacophores. The demonstration that the three identified compounds inhibit LTA4H-h and hnps-PLA2 simultaneously validates our strategy and the hypothesis that compounds having binding conformations matching the common pharmacophores in multiple proteins are potential inhibitors of multiple protein targets. Given this hypothesis, pharmacophore searches in small molecule databases can be performed to identify probable candidates. The common pharmacophores also provide guidance for designing a combinatorial library in which different fragments matching single pharmacophore can be combined to construct active compounds. On the other hand, this strategy can also be extended to search existing single-target inhibitors and disclose their possible multiple functions. For example, on the basis of the common pharmacophores, we searched inhibitors reported for LTA4H-h.[19,21,29−36] About 350 LTA4H-h inhibitors with diversified chemical structures were analyzed, and the conformations of several compounds (shown in Figure S1 in the Supporting Information) were found to match the common pharmacophores well. For the convenience of synthesis, a similar compound (compound **11** in Chart 1) of an inhibitor (compound **10**[14] in Chart 1) was synthesized. Compound **11** showed great potency in inhibiting the aminopeptidase activity and the epoxide hydrolase activity of LTA4H-h with IC_{50} of 228 ± 14 and 35 ± 2 nM, respectively. At the same time, compound **11** inhibited the activity of hnps-PLA2 with an IC_{50} of 7.3 ± 1.0 μM. Further work on multitarget inhibitor design is underway.

Conclusion

In the new phase of reevaluating the target-focused ligand discovery model in medicinal chemistry, multitarget inhibitors provide a new opportunity for treating complex diseases. How to rationally design efficient multitarget inhibitors is critical. To solve this problem, we proposed a novel strategy that combined receptor-based common pharmacophores with molecular docking. After deriving the pharmacophores from the binding pockets of different proteins using the Pocket v.2 program, common pharmacophores were identified. Then the binding conformations of small molecules were predicted by molecular docking, and small molecules whose binding conformations accommodated the common pharmacophores were selected as candidates to bind with multiple targets. Three compounds identified through this strategy inhibited LTA4H-h and hnps-PLA2 simultaneously, validating the feasibility of the strategy. The common pharmacophores not only can provide guidelines for pharmacophore searches in small-molecule databases or collections of known inhibitors,but can also be applied in combinatorial library design for further compound development.

Experimental Section

Molecular Modeling Methods. Target Structure Preparation. In this modeling study, we used the complex structure (PDB code 1hs6) of human LTA4H-h with 2-(3-amino-2-hydroxy-4-phenylbutyrylamino)-4-methylpentanoic acid (bestatin)[16] and the structure (PDB code 1db4) of hnps-PLA2 with [3-(1-benzyl-3-carbamoylmethyl-2-methyl-1*H*-indol-5-yloxy)propyl]phosphonic acid (indole **8**).[17] Protein structure files were prepared by the molecular modeling program Sybyl 6.91, and all heteroatoms except the metals (zinc in LTA4H-h, calcium in hnps-PLA2) were removed.

Pharmacophore Derivation. The pharmacophores were generated by Pocket v.2,[15] a program that derives pharmacophore models directly from given protein−ligand complex structures without human intervention. Ligands in the complexes (bestatin in LTA4H-h, indole **8** in hnps-PLA2) were extracted, and hydrogen atoms were added. The 3-D structures of LTA4H-h and hnps-PLA2 in PDB format and the corresponding ligands in Mol2 format were used as input files for Pocket v.2 to derive pharmacophore models; the key features in the model were automatically reduced to a reasonable number. On the basis of a comparison between pharmacophores generated from LTA4H-h and hnps-PLA2, common pharmacophores were identified.

Binding Mode Prediction for Small Molecules in Databases. To search for compounds that accommodated the common pharmacophores in the ACD (MDL Available Chemical Database, release ACD 2002.2), it was necessary to predict the compounds' binding conformations in both proteins. First, the binding conformations of the ACD compounds with LTA4H-h were predicted using the Dock 4.0 program,[37] which is known for its speed in flexible docking. Gasteiger−Hückel charges were added to the compounds in ACD. Hydrogen and partial charges based on the AMBER force field were assigned to LTA4H-h using the Sybyl 6.91 program as required in DOCK 4.0. A Connolly surface of the binding pocket was generated using DMS with a probe radius of 1.4 Å. The SPHGEN program was used to create overlapping spheres complementary to the protein surface. The binding pocket included all receptor residues within a distance of 6 Å from any atom of the reference ligands. Dock 4.0 was run using the MPI edition, modified in our laboratory, on a Linux Cluster Platform that contains 128 CPUs (Inter P-IV 2.6 GHz) on 64 computing nodes. A maximum of 150 orientations for the anchor fragment and a manual matching mode with 0.5 Å distance tolerance were applied. To speed up the calculations, 50 configurations per cycle were set for the growth of the ligands and simplex minimization was applied to each growth step of the ligands. The conformations with the lowest docked energy were assumed to be the binding conformations of the small molecules in the protein pocket.

Compound Filtering. Because of the low affinity of multitarget inhibitors, the binding conformations of the top 150 000 compounds (60% of the database) ranked by docking energy-score in Dock 4.0 were extracted and were used to check whether their binding conformations with LTA4H-h accommodated the common pharmacophores by Pscore, an in-house program developed in our laboratory to check whether the binding conformations of compounds in the binding pocket accommodate the designated pharmacophores. Compounds that matched the common pharmacophores were analyzed in further steps.

Binding Conformation Prediction in Multiple Proteins. We used Autodock 3.05 to check the binding conformations of the chosen compounds in LTA4H-h and hnps-PLA2 more accurately. Grid maps of 60 × 60 × 60 and of 40 × 40 × 40 grid points, centered on the ligands of the complex structures, were used for LTA4H-h and hnps-PLA2, respectively, to cover the binding pockets. A spacing of 0.375 Å was set, and a Lamarckian genetic algorithm was used for all molecular docking simulations. Population size of 300, mutation rate of 0.02, and crossover rate of 0.8 were set as the parameters. Simulations were performed using up to 2.5 million energy evaluations with a maximum of 27 000 generations. Each simulation was performed 100 times, yielding 100 docked conformations. The lowest energy conformations were regarded as the binding conformations between the ligands and the proteins.

Candidate Selection. After the binding conformations were predicted by Autodock 3.05, compounds with conformations in all the expected proteins accommodating the common pharmacophores were selected again by Pscore. However, pharmacophores are only the basic features responsible for binding, and the actions of other atoms and groups that are not reflected in the pharmacophores also need to be considered. Finally, selected compounds that were commercially available were bought to test for activity against LTA4H-h and hnps-PLA2.

Activity Assay. Inhibition Assay of Aminopeptidase Activity of LTA4H-h. The aminopeptidase activity was determined spectrophotometrically by the release of the colorimetric product *p*-nitroanilide (p-NA) from Ala-*p*-nitroanilides. The enzyme (1−20 μg) was incubated at 37 °C in 96-well microtiter plates in 50 mM Tris-HCl, pH 8.0, containing 100 mM NaCl. The reactions were initiated by the addition of Ala-*p*-nitroanilides as substrate. The formation of p-NA was then subsequently monitored at 37 °C for 15 min at 405 nm using a multiwell ultraviolet spectrometer (Spectra Max 190, Molecular Devices). For the IC_{50} determinations, 0.125 mM substrate was used. All of the reactions containing inhibitors were performed after incubating inhibitors with enzymes for 2 min. The final concentration of DMSO was 5% (v/v). The amount of p-NA released from proteolysis was calculated with a standard curve generated using analytical grade p-NA. The initial reaction rates at different inhibitor concentrations were used for calculating the IC_{50} values.

Inhibition Assay of Hydrolase Activity of LTA4H-h. The epoxide hydrolase activity was determined using an ELISA assay to quantify the amount of LTB_4. LTA_4 methyl ester (Cayman Chemical) was hydrolyzed in cold acetone with 50 mM NaOH (20%, v/v) under an inert atmosphere of nitrogen at 22 °C for 40 min. The resulting basic solution of LTA_4 was stored at −80 °C and diluted directly into buffers when used. For the determination of the effect of small molecules on the epoxide hydrolase activity of LTA4H-h, 300 ng of enzyme was incubated with compounds in 180 μL of reaction buffer (10 mM sodium phosphate, pH 7.4, 4 mg/mL BSA, 5% v/v DMSO) for 15 min at 37 °C. The reaction was initiated by the addition of 20 μL of LTA_4 (150 nM final assay concentration) and incubated for another 10 min at 37 °C. A 25 μL sample was added to 500 μL assay buffer without DMSO to stop the reaction and then further diluted 5 times. LTB_4 was quantified in the diluted sample by a commercially available LTB_4 ELISA kit (Cayman Chemical).

Inhibition Activity Assay of hnps-PLA2. The assay for the effects of chosen compounds on hnps-PLA_2 was carried out in a 96-well plate using 1-anilinonaphthalene-8-sulfonate (ANS) as an "interfacial probe". First, 50 μL of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) solution (40 mM in methanol) and 50 μL of deoxycholic acid (40 mM in methanol) were mixed and quickly pipetted into doubly distilled water (1 mL). The solution was stirred for 1 min, sonicated in a water bath for 2 min, and used as the stock substrate solution. Then 150 μL of reaction buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM NaN_3, 5 μg/mL bovine serum albumin (BSA), and 10 mM ANS), 20 μL of substrate stock solution, 10 μL of $CaCl_2$ stock solution (100 mM), and 10 μL of inhibitor stock solution (dissolved in DMSO) were incubated at 25 °C for 10 min. Reactions were started by adding 10 μL of hnps-PLA2 stock solution (3.2 μg/mL) and monitored by excitation at 377 nm and emission at 470 nm utilizing a multiwell fluorometer (SpectraMax GeminiXS, Molecular Devices). Fluorescence signals were recorded for 20 min with a kinetics mode program. The initial reaction rates at different inhibitor concentrations were used for calculating the IC_{50} value.

The IC_{50} value was calculated with the following equation:

$$\frac{V_0}{V} = 1 + \frac{[\mathrm{I}]}{\mathrm{IC}_{50}}$$

where V_0 is the initial enzyme reaction rate without inhibitor, V is the initial enzyme reaction rate with the inhibitor at various concentrations, [I] is the concentration of the inhibitor, and IC_{50} is the concentration giving 50% inhibition. IC_{50} values were obtained from at least three sets of experiments.

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Supporting Information Available: A listing of MDL number in the ACD database for the compounds tested, a listing of 163 compounds whose binding conformations with LTA4H-h matched the common pharmacophores, and plots of IC_{50} data and inhibition data. This material is available free of charge via the Internet at http://pubs.acs.org.

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