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De Novo Design of Multitarget Ligands with an Iterative Fragment-Growing Strategy

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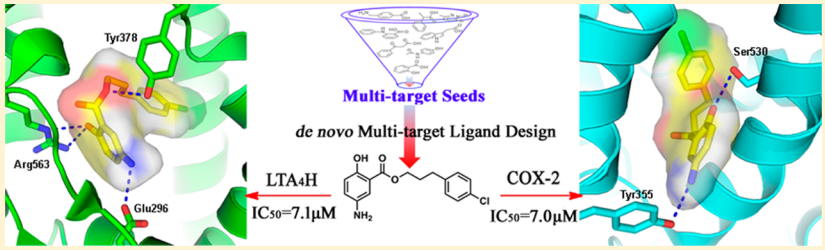
Supporting Information



ABSTRACT: The discovery of multitarget drugs has recently attracted much attention. Most of the reported multitarget ligands have been serendipitous discoveries. Although a few methods have been developed for rational multitarget drug discovery, there is a lack of elegant methods for *de novo* multitarget drug design and optimization, especially for multiple targets with large differences in their binding sites. In this paper, we report the first *de novo* multitarget ligand design method, with an iterative fragment-growing strategy. Using this method, dual-target inhibitors for COX-2 and LTA_4H were designed, with the most potent one inhibiting PGE_2 and LTB_4 production in the human whole blood assay with IC_{50} values of 7.0 and 7.1 μM, respectively. Our strategy is generally applicable in rational and efficient multitarget drug design, especially for the design of highly integrated inhibitors for proteins with dissimilar binding pockets.

INTRODUCTION

Single-target drugs are often less effective in controlling complex diseases with multiple pathogenic factors, such as diabetes, inflammation, cancer, and CNS disorders.[1−3] Multi-target drugs, which are able to interact with several drug targets simultaneously, lead to new and more effective medications for a variety of complex diseases, even with relatively weak activities.[4,5] Consequently, much attention has been given to multitarget drug design, and methods for multitarget ligand discovery were developed accordingly.[6,7] Among them, framework combination and screening are two widely used approaches to multitarget lead generation. However, framework combination usually produces ligands with large molecular weights and low ligand efficiencies with possible poor oral pharmacokinetics. Moreover, the chance of screening success is generally low, especially for unrelated targets where the pharmacophores are distinct.[8] Therefore, the efficient discovery of "highly integrated" ligands for unrelated targets remains challenging, and a general strategy for multitarget rational drug design for dissimilar targets needs to be developed.

LigBuilder 2 is a *de novo* drug design program developed in the authors' lab with embedded synthesis accessibility analysis and various scoring schemes.[9] We recently upgraded the program to LigBuilder 3 and developed a new function to elegantly and simultaneously design and optimize molecules for multiple targets.[10]

Cyclooxygenase (COX)/prostaglandin E_2 synthases (PGES) and 5-lipoxygenase (5-LOX)/leukotriene A_4 hydrolase (LTA_4H) are the two major metabolic pathways that produce inflammatory mediators from arachidonic acid (AA).[11,12] Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in anti-inflammatory therapy. However, commonly used NSAIDs are reported to associate with gastrointestinal side effects due to their inhibition of the synthesis of the prostaglandins PGI_2 and PGF_2.[13] Although selective COX-2 inhibitors (e.g., coxibs) have a lower risk of gastrointestinal damage, they exert cardiovascular side effects presumably by altering the balance between PGI_2 and thromboxanes.[14] Dual-target inhibitors for COX-2 and LTA_4H are considered to be able to establish a more efficient anti-inflammatory therapy strategy with reduced side effects.[15,16] Until now, only a few COX-2/LTA_4H dual-target inhibitors have been reported.[13] A possible difficulty is due to the large difference in substrate-binding pockets between the two enzymes.

In the present study, COX-2 and LTA_4H were used as the paradigm. We used our *de novo* multitarget drug design method

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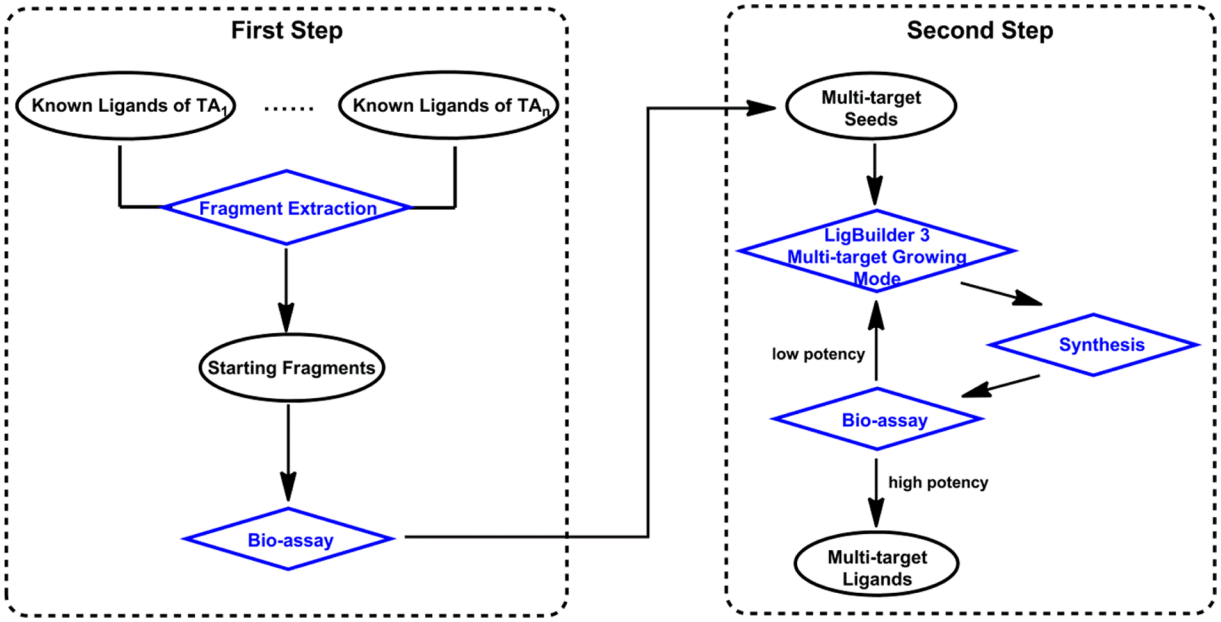
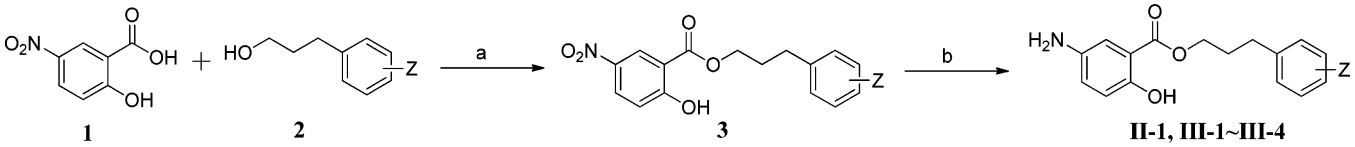


Figure 1. Flowchart of *de novo* multitarget ligand design method.

Scheme 1. Synthesis of Compounds II-1 and III-1 through III-4[a]



[a]Reagent and conditions: (a) EDCI, DMAP, THF, rt; (b) H_2, Pd/C, MeOH or AcOEt.

with an iterative fragment-growing strategy to design dual-target inhibitors for both proteins. Representative compounds were synthesized, and their inhibition activities to purified enzymes and human whole blood were tested. Surface plasmon resonance was used to confirm the direct binding interactions of the serial optimized compounds with COX-2 and LTA_4H.

METHODS

Overview. The overall strategy is shown in Figure 1. In order to increase the success rate of *de novo* multitarget drug design, an iterative fragment-growing strategy was used in our design process. This strategy was borrowed from fragment-based drug discovery (FBDD), which has emerged over recent years as an effective drug discovery strategy.[17−21] The main idea in FBDD is to start the hit and lead optimization process with small molecular fragments with molecular weights in the 120−300 Da range.[22] The molecular complexity model proposed by Hann and co-authors shows that smaller ligands are more likely to bind to multiple targets than larger ones.[23] In the first step, we designed small fragments with limited molecular weights. The activities of these fragments to COX-1/2 and LTA_4H were tested with high-concentration biochemical assays, and the potent fragments with dual inhibition to the both targets were selected. These potent fragments, named "multitarget seeds", were used as starting structures in the following optimization. In the second step, using LigBuilder 3, the multitarget seeds grow larger with molecular weights increasing within a limited range, and we experimentally selected the more potent ones as seed structures for the next round. This "design−synthesis−bioassay" loop was repeated for several rounds to ensure successful design ligands with high potencies for multiple targets.

Target Protein and Fragments Preparation. The crystal structure of COX-2 (PDB code 1PXX) and LTA_4H (PDB code 1HS6) were downloaded from the Protein Data Bank.[24] Water molecules and co-crystallized ligands were removed from the structures using the SYBYL software.[25] The structures of all the small molecule fragments were modeled using the SYBYL software and optimized with Tripos force field.[25]

Fragments Docking. AutoDock 4.0[26] was used to dock the fragments hits into the binding site of the two enzymes. For COX-2, the size of docking box is 21.0 Å × 14.0 Å × 17.0 Å, with the grid spacing of 0.375 Å. For LTA_4H, the size of the docking box is 31.0 Å × 19.0 Å × 23.0 Å, with a grid spacing of 0.375 Å. Gasteiger charges were used for both the protein and ligand structures. The Lamarckian genetic algorithm (GA) was used, and the related AutoDock parameters were set as follows: 200 GA runs, each with population size of 200; 25 million energy evaluations; and a maximum of 270,000 generations per GA run. The 200 independent GA runs from AutoDock were processed using the built-in clustering analysis with a 2.0 Å cutoff.

Multitarget Ligand Design. Liguilder 3 was used to produce novel compounds based-on the seed structures. Ligands generated by LigBuilder 3 are expected to bind to both proteins with different binding conformations. The drug-like and privileged building blocks used in this work were inherited from LigBuilder 2. We apply the "Growing Mode" of Liguilder 3 to manipulate the optimization process. The GA parameters were set as follows: GA population size of 5000, GA

parent ratio of 10%, GA generation number of 15. To enhance the diversity and quality of the designed compounds, we designed about 1 million molecules and then apply the "Recommendation" module of LigBuilder 3 to pick the top 1000 results in each round of optimization. Considering each GA procedure will produce hundreds of compounds, this procedure was repeated more than 1000 times, which ensures that each seed structure in the seed list has been used.

Chemistry. Compounds **II-1** and **III-1** through **III-4** were prepared in moderate to high yields (Scheme 1). 5-Nitrosalicylic acid reacted with different substituted 3-phenylpropyl alcohols, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI) was used as a coupling agent in the presence of a catalytic amount of 4-dimethylaminopyridine (DMAP). The synthesis of the first round compounds and experimental details are supplied in the Supporting Information.

***In Vitro* Enzymatic Assay.** The title compounds were evaluated in enzymatic assay of COX-1/2 and LTA_4H. The enzyme activity of the purified COX-1/2 was measured by a chromogenic assay based on monitoring the absorption of oxidized N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) during the reduction of PGG_2 to PGH_2 at 610 nm.[27] The LTA_4H hydrolase activity was measured using an ELISA assay kit to quantify the amount of LTB_4 generated.

Human Whole Blood Assay. **II-1** and **III-1** were also evaluated in human whole blood assay. In this study, *E. coli* liopolysaccharide (LPS) was used to induce the COX-2/PGES pathway in human whole blood, and A23187 was used to induce the 5-LOX/LTA_4H pathway (see Surpporting Informmation for details).[28,29]

Surface Plasmon Resonance (SPR) Analysis. SPR experiments were performed on a Biacore T200 using a CM5 sensor chip. PBS-P+ 10× buffer of GE Healthcare was diluted to running buffer with 5% DMSO. The immobilization levels were 10,000−15,000 RU. The references were blocked with ethanolamine-HCl after activated with EDC and NHS. A small molecule wizard of T200 was used to test the binding of **SH-1** and the following optimized compounds. The binding strengths of these compounds at 100 μM were tested for comparison. The same method was used to the measure of kinetic profiles of **III-1** with the two targets (2-fold dilution series, five concentrations), and a 1:1 interaction model was used to fit the analysis data.

RESULTS

Dual-Target Seeds Identification. LigBuilder 3 is a *de novo* multitarget design method. It can start with lead structures for multitargets as seeds and perform the optimization process until larger molecules with high potencies for each target are designed. If there are no lead structures available, LigBuilder 3 can generate lead structures automatically. However, small fragments from known ligands of single targets are more reliable to become lead structures than random ones. As many single-target inhibitors of COX-2 and LTA_4H have already been discovered, we extracted small fragments from these inhibitors, which can also be done by LigBuilder 3 automatically. In this fragment extraction and selection process, considering Congreve's rule-of-three,[30] we employed several rules to select known ligands. (1) The molecular weight of the screening fragments should be no more than 300 Da. (2) The number of hydrogen bond acceptors and donors should be less than or equal to three. (3) The number of rings should be greater than or equal to one. (4) The structure−activity relationship (SAR) of known ligands with their targets was manually considered. Consequently, 21 fragments from the extracted fragment library were selected and bought from chemical reagent corporations. Among them, 16 fragments were derived from known COX-1/2 inhibitors, and the other five were derived from known LTA_4H inhibitors (see Table S-1, Supporting Information, for these fragment structures). As the fragments were small and their potencies were expected to be low, high-concentration biochemical assays, typically 250−1000 μM,[31] were used to identify active fragment hits. In our study, the inhibition activity of these fragments to COX-1/2 and LTA_4H were measured at 1 mM concentrations, and nine dual functional hits were found. The high hit rate (43%) confirmed that extracting small fragments from known single-target inhibitors is a reliable method to build starting seeds for multitarget ligand design.

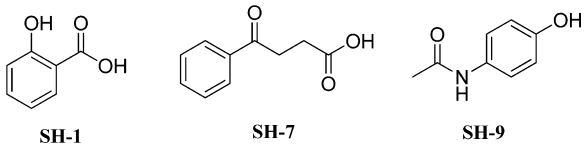


Figure 2. Structures of **SH-1**, **SH-7**, and **SH-9**.

Seeds Growing. After discovering multitarget lead fragments as seed structures, LigBuilder 3 was used to optimize these seeds in a stepwise way. The lead fragments were docked into each of the two targets, and several possible docking conformations were selected for further optimization. As it is difficult to predict the precise binding modes for "small" fragments in the binding sites of the targets, selecting multiple binding conformations is essential for follow-up optimization. To ensure that the optimization direction was reasonable, for each round, the molecular weight increase was restricted to within 100 Da in the fragment growing process. The designed ligands were selected for synthesis and bioassay evaluation, and the active ligands were subject to the next round of optimization. Through several rounds optimization, potent dual target ligands were expected be discovered.

First Round of *de Novo* Multi-Target Optimization. AutoDock 4.0 was used to dock the nine lead fragments into COX-2 and LTA_4H (PDB: 1pxx/COX-2 and 1hs6/LTA_4H). The docking conformations with the lowest energies and the representative conformations of the five largest clusters were selected as seeds for LigBuilder 3 growing. Duplicates caused by the five largest clusters containing the lowest energy conformations were removed, and 50 conformations were selected for each receptor in this step. Using the 50 conformations of the nine lead fragments as seeds for both receptors, LigBuilder 3 produced 1.1 million compounds and exported 1000 top-ranked compounds. Analysis of these molecules showed that the derivatives of three "single ring" lead fragments, **SH-1**, **SH-7**, and **SH-9** (Figure 2) were in the 1000 top-ranked designed molecules, while the derivatives of the other fragments were ranked beyond the top 1000 designed molecules. Three criterions were used to choose compounds for the experimental testing: (1) structures with "minimum modification"', (2) "common frameworks" from a series of analogues, and (3) structures that are easily prepared. Consequently, six representative exported molecules of **SH-1**,

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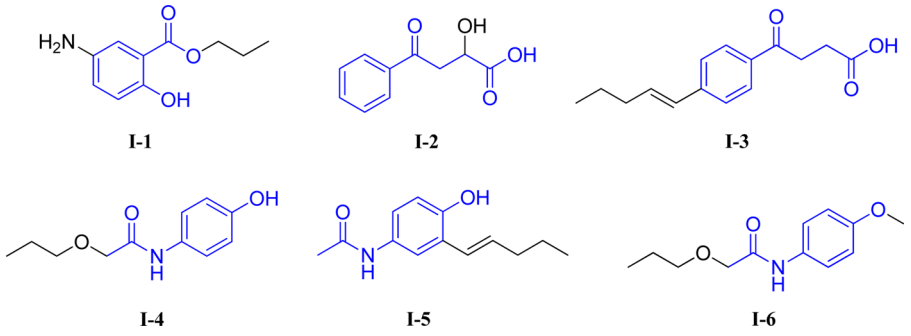


Figure 3. Ligands selected from first-round optimization (fragments in blue are parent frameworks).

Table 1. COX-1/2 and LTA_4H Inhibition Rates of First-Round Optimized Ligands at 100 μM

compound	inhibition % COX-1	inhibition % COX-2	inhibition % LTA_4H
SH-1[a]	13 ± 5	12 ± 1	48 ± 21
I-1	24.0 ± 2.0	14.6 ± 1.6	17.5 ± 1.0
I-1[b]	92.4 ± 1.0	98.3 ± 1.2	43.2 ± 4.4
I-2	n. i.[c]	n. i.	32.0 ± 2.5
I-3	n. i.	n. i.	35.0 ± 1.5
I-4	n. i.	n. i.	n. i.
I-5	n. i.	n. i.	70.3 ± 0.8
I-6	27.3 ± 1.5	15.2 ± 2.2	n. i.

[a]**SH-1**was measured at 1 mM. [b]**I-1** was measured at 500 μM. [c]n.i. = no inhibition.

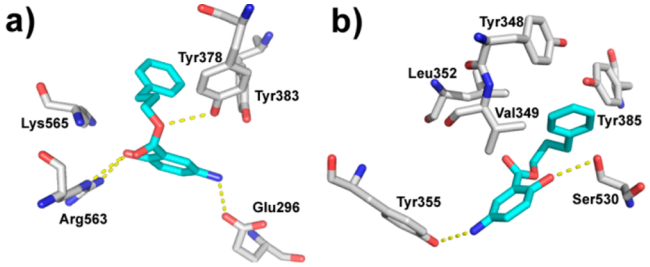


Figure 4. Designed poses of **II-1** with (a) LTA_4H and (b) COX-2.

SH-7, and **SH-9** derivatives were selected for synthesis and bioassay (Figure 3).

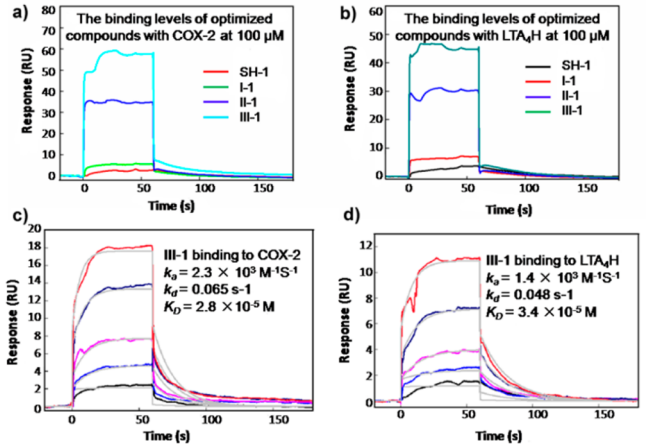
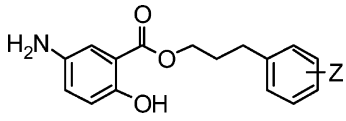


Figure 5. SPR binding curves. Binding levels of optimized compounds with COX-2 (a) and LTA_4H (b) at 100 μM. Interaction profiles of III-1 with COX-2 (c) and LTA_4H (d).

The *in vitro* enzymatic assays of the two targets (see Supporting Information for details) were used to evaluate the first-round optimized ligands, and the compound **I-1**, which was evolved from **SH-1**, shows dual target inhibition activity against COX-2 and LTA_4H (Table 1). Therefore, using **I-1** as the seed, second-round optimization with LigBuilder 3 was performed.

The effective binding modes of **I-1** with the two targets are not evolved from the lowest energy conformations of the **SH-1** docking results. Consequently, the use of multiple different

Table 2. Bio-Evaluation of the Title Compounds



		in vitro IC_{50} (μM)		HWB IC_{50} (μM)	
compound	Z	COX-2	LTA_4H	PGE_2	LTB_4
ref-1[a]	–	–	0.025 ± 0.002	–	0.63 ± 0.17
Flur[b]	–	0.018 ± 0.005	–	4.3 ± 1.2	–
5a[c]	–	7.7 ± 0.3	0.68 ± 0.27	8.4 ± 0.7	6.9 ± 0.1
II-1	H	56.0 ± 8.0	16.6 ± 0.7	18.0 ± 1.8	12.6 ± 0.5
III-1	4-Cl	16.8 ± 3.4	7.2 ± 1.3	7.0 ± 2.5	7.1 ± 0.7
III-2	4-F	>50	1.7 ± 0.2	n.t.[d]	n.t.
III-3	4-OMe	>50	3.0 ± 0.1	n.t.	n.t.
III-4	3-F-4-Cl	76.5 ± 0.1	>50	n.t.	n.t.

[a]Positive control of LTA4H. [b]Positive control of COX-2. [c]The best dual-target inhibitor of COX-2 and LTA_4H reported.[15] [d]n.t.= no test.

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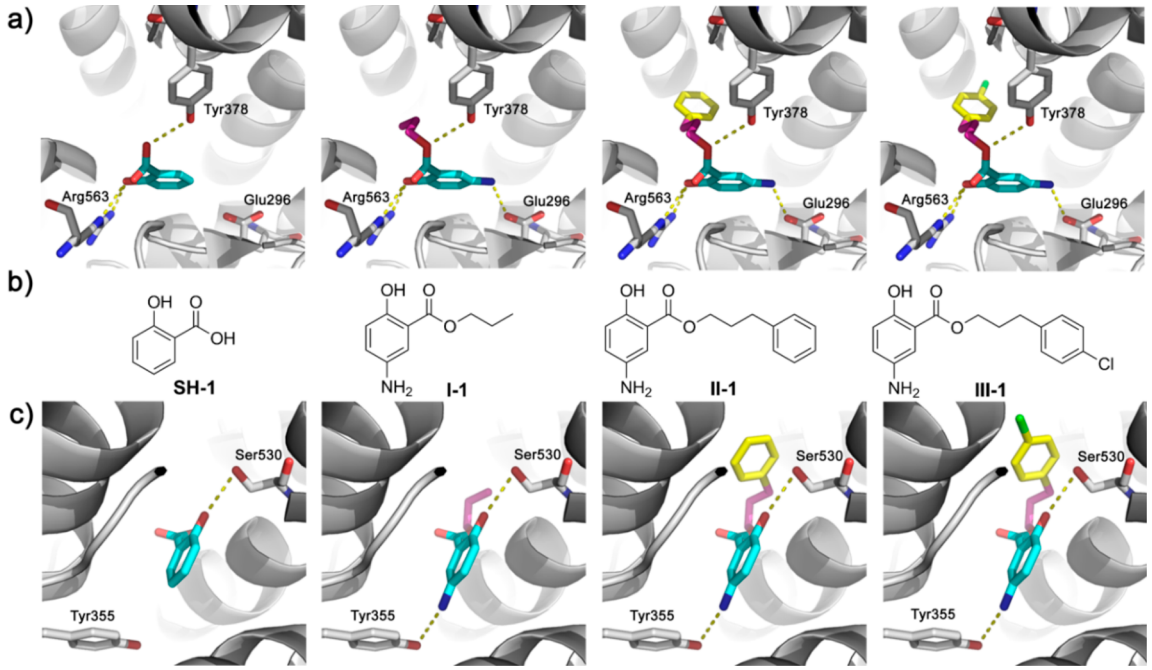

Figure 6. Optimization path of **SH-1**. (a) Designed poses of LTA_4H with **SH-1**, **I-1**, **II-1**, and **III-1**. (b) Structure of **SH-1**, **I-1**, **II-1**, and **III-1**. (c) Designed poses of COX-2 with **SH-1**, **I-1**, **II-1**, and **III-1** (generated by LigBuilder 3).

docking conformations of each fragment hit as a starting point for LigBuilder 3 is vital for the success of *de novo* multitarget ligand design. LigBuilder 3 was developed to have a strong ability to simultaneously design and optimize multiple ligands with multiple binding poses.

Second-Round of *de Novo* Multi-Target Optimization. All binding modes of **I-1** with the both targets in the first round optimization were used as seeds in this round. In this round, the ligands became larger, so the log *P* value was restricted from −1.4 to 6.6, which is a slight extension of the qualifying range (−0.4 to 5.6) suggested by Ghose[32] for further derivatization. For the same reason, the number of hydrogen bond acceptors and donors was set to less than five. For the genetic algorithm in LigBuilder 3, the number of genetic algorithm runs was set to 15, and population size was set to 3000. One million molecules were produced with LigBuilder 3 and ranked by size-independent ligand efficiency as proposed by Nissink,[33] and the 1000 top-ranked compounds were then exported. Analysis of the designed molecules shows that a common framework 3-phenylpropyl-5-amino-2-hydroxybenzoate (**II-1**) can represent all the key interactions of these molecules to both targets (Figure4); hence, **II-1** was selected for experimental verification. We synthesized **II-1** and measured its inhibition activities toward the two enzymes. The IC_{50} values of **II-1** to the two targets were measured in the *in vitro* enzymatic and the human whole blood (HWB) assays (Table 2). Two known inhibitors, 1-(2-(4-phenoxyphenoxy)ethyl)pyrrolidine (**ref-1**),[34] a reported LTA_4H inhibitor, and fluorobiprofen (**Flur**),[35] a reported COX-2 inhibitor, were used as positive controls (see Supporting Information for the structures of **ref-1** and **Flur**).

Further Improve the Potency of II-1. As revealed in Figure 4, both the hydroxyl group and amino group are engaged in the key hydrogen bonds, which anchor the molecules in the binding sites. The three-carbon flexible linker between the polar moiety and hydrophobic moiety is appropriate for the interaction of **II-1** with both the targets, allowing **II-1** to adopt different conformations in the two binding sites. Thus, four derivatives of **II-1** with different hydrophobic phenyls were designed and synthesized. Table 2 shows the structures and *in vitro* activities of these derivatives. The 4-chlorophenyl derivative **III-1** possesses improved activity to both targets (IC_{50} values: 16.8 μM to COX-2 and 7.2 μM to LTA_4H). Although the 4-fluorophenyl derivative **III-2** and the 4-methoxyphenyl derivative **III-3** showed improved inhibition activities for LTA_4H, their inhibition activities for COX-2 disappeared. On the other hand, the 4-chloro-3-fluorophenyl derivative **III-4** shows comparable COX-2 inhibition activity, but loses LTA_4H inhibition activity. Consequently, **III-1**, as a more potent COX-2 and LTA_4H dual-target inhibitor, was found.

Prontein−Ligand Binding Assay. In order to confirm the interactions of the serial optimized compounds with both COX-2 and LTA_4H, the binding strengths of these compounds were tested using SPR at 100 μM concentration, and the dissociation constants of the best compound **III-1** with the both targets were measured too. As shown in Figure 5a and b, the binding strengths increased with each round of optimization. The dissociation constants of **III-1** were 28 μM for COX-2 and 34 μM for LTA_4H (Figure 5c and d).

■ DISCUSSION

The optimization from **SH-1** to **III-1** is showed in Figure 6. In the binding mode of **SH-1** with the two targets, the hydroxyl and carboxyl groups interact with the two targets as hydrogen bond acceptors, and the H of the carboxyl group is free. Hence, the displacement of H with an alkyl group retains the activity of the parent structure. Moreover, the amino group introduced during first-round optimization makes hydrogen bonds with Tyr365 of COX-2 and Arg563 of LTA_4H (Figure 4), resulting in an activity increase of **I-1** toward both enzymes (Table 1). In the second round of optimization, the newly added phenyl group leads to a notable inhibition increase in **II-1** to both the

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targets due to the hydrophobic interaction between the phenyl group and the two binding sites (Figure 4). Hence, using a *de novo* multitarget design method, a high quality dual-target inhibitor hit **II-1** was designed efficiently, and a more potent **III-1** was found after a simple modification of **II-1**. **III-1** inhibits PGE_2 and LTB_4 production in the human whole blood assay with IC_{50} values of 7.0 and 7.1 μM, respectively. The SPR binding assay confirms that the inhibition activities of these ligands are related to their direct binding to the targets.

The COX-2 and COX-1 selectivity indicator (SI = IC_{50} (COX-1)/IC_{50} (COX-2)) is one of the main evaluation indicators for the safety of anti-inflammatory drugs. In previous work, we built a mathematical model for the inflammation-related arachidonic acid metabolic network and predicted that the use of COX-2 and LTA_4H dual-target inhibitors with COX-2 selectivity (SI approximately 7) is an optimum intervention solution. We tested the inhibition activity of **III-1** to COX-1 (179 ± 21 μM) and found that its selectivity for COX-2 is 11.1, comparable to the ideal value. With good potency in both *in vitro* and HWB assays as well as COX-2 selectivity, **III-1** is a promising lead compound for further development of novel anti-inflammatory drug.

Compared to framework combination methods using molecular linking, substitution, or fusion strategies, LigBuilder 3 builds highly efficient multifunctional ligands. Ligands are designed to interact with different targets with distinct conformations, and atoms of the ligands are designed to maximize their interaction with all the targets.

CONCLUSIONS

A *de novo* multitarget drug design method, with a special iterative strategy to improve the design success rate, was developed. This strategy is especially for the design of highly integrated inhibitors for proteins with dissimilar binding pockets. Using this method, the best in class dual target inhibitor for COX-2 and LTA_4H was discovered. The successful application of our strategy shows that is a powerful tool for multitarget ligand design and optimization. This method can be generally used to design and optimize multifunction ligands binding to two or more targets.

ASSOCIATED CONTENT

Supporting Information

Fragments library, synthesis, bioassay, and scanned spectrum of the ^1H NMR and ^{13}C NMR data of representative compounds. 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.

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ABBREVIATIONS

NSAIDs, nonsteriodal anti-inflammatory drugs; FBDD, fragment-based drug design; COX, cyclooxygenase; 5-LOX, 5-lipoxygenase; PGES, prostaglandin E synthase; LTA_4H, leukotriene A4 hydrolase; PGE_2, prostaglandin E_2; LTB_4, leukotriene B_4

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