Discovery of Leukotriene A4 Hydrolase Inhibitors Using Metabolomics Biased Fragment Crystallography†

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We describe a novel fragment library termed fragments of life (FOL) for structure-based drug discovery. The FOL library includes natural small molecules of life, derivatives thereof, and biaryl protein architecture mimetics. The choice of fragments facilitates the interrogation of protein active sites, allosteric binding sites, and protein–protein interaction surfaces for fragment binding. We screened the FOL library against leukotriene A4 hydrolase (LTA4H) by X-ray crystallography. A diverse set of fragments including derivatives of resveratrol, nicotinamide, and indole were identified as efficient ligands for LTA4H. These fragments were elaborated in a small number of synthetic cycles into potent inhibitors of LTA4H representing multiple novel chemotypes for modulating leukotriene biosynthesis. Analysis of the fragment-bound structures also showed that the fragments comprehensively recapitulated key chemical features and binding modes of several reported LTA4H inhibitors.

Introduction

The wealth of data deposited by structural genomics initiatives indicates that ~20% of protein crystal structures contain stably bound small molecules. These endogenous ligands include substrates, cofactors, products, bound metal ions, and even components of the crystallant. For example, of ~3500 structures deposited by the Protein Structure Initiative (as of January 2009), approximately 342 contain natural ligands and 280 contain cofactors (http://smb.slac.stanford.edu/jcsg/Ligand_Search/). Not infrequently, electron density deriving from an unknown ligand is present in deposited structures. Moreover, there are numerous reports of natural molecules that mediate powerful biological effects by stabilizing protein–protein interactions. Evolution produced conserved protein motifs that optimally bind small molecules representing preferred or privileged chemical architectures. Kuntz et al. showed empirically that for ligands that bind strongly to proteins, each non-hydrogen atom contributes about ~1.5 kcal/mol to the free energy of ligand binding to about 15 non-hydrogen atoms (∼180 MW), the maximum size of most enzyme substrates or metabolites. Furthermore, the dominant interactions were van der Waals interactions that will depend upon shape complementarity and hydrophobic effects. Thus, the coevolution of protein structures with chemical architectures represented in the metabolome will select for optimization of van der Waals interactions based on shape complementarity. These preferred chemical architectures will bind proteins with high ligand efficiency. This suggests that the natural molecules of life represented by the metabolome can be a preferred starting point for fragment based drug discovery (FBDD).

We therefore constructed a screening set for FBDD that we have called “fragments of life” (FOL). Fragment-based drug discovery has gained acceptance within the pharmaceutical industry (as reviewed by Congreve et al.), as it provides an alternative to expensive and sometimes inefficient high-throughput screening (HTS) methods for chemical hit identification. The general concept of FBDD involves screening of low molecular weight “rule of three” compounds (MW < 300, ≤3 rotatable bonds, ≤3 hydrogen bond donor/acceptor(s), TPSA < 60, ClogP < 3) against target macromolecules using a variety of detection methods. These include X-ray crystallography, nuclear magnetic resonance (NMR), surface plasmon resonance, differential thermal denaturation, fluorescence polarization, and other techniques.

†This manuscript describes structural information from 20 unique LTA4H structures with bound ligands, all of which have been entered into the Protein Data Bank and listed here in the following format (compound number bound to LTA4H, PDBID code): 2, 3FTS, 3, 3FU; 3 + 1, 3FTX; 4, 3FTV; 4 + acetate, 3FTW; 5, 3FTY; 6, 3FUO; 7, 3FU3; 8, 3FU5; 9, 3FU6; 10, 3FU2; 11, 3FUH; 12, 3FUE; 13, 3FU; 14, 3FU1; 15, 3FU; 16, 3FUK; 17, 3FU; 18, 3FU; 19, 3FHT.

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‡Abbreviations: LTA4H, leukotriene A4 hydrolase; LCMS, liquid chromatography mass spectrometry; FOL, fragments of life; FBDD, fragment based drug discovery; TPSA, total polar surface area; NMR, nuclear magnetic resonance; PDB ID, Protein Data Bank identification number; ClogP, calculated log of octanol/water partition coefficient; LTB4, leukotriene B4; FOL-Nat, members of the fragments of life library that are natural molecules of life; FOL-NatD, members of the fragments of life library that are synthetic derivatives or isosteres of natural molecules of life; FOL-Biaryl, members of the fragments of life library that are synthetic biaryl molecules, many of whose energy minimized structures mimic α-, β-, and γ-turns; HWB, human whole blood; rt, room temperature.
The design of reasonably sized fragment libraries is essential for successful FBDD. Considering both the concepts of ligand efficiency and the size of feasible chemical space for fragments, we aimed to establish a compact fragment set (~1300 molecules) that (i) encompasses diverse biological and chemical thought, (ii) facilitates chemical optimization, and (iii) has solubility properties that make the library compatible with rapid screening methods (Figure 1). We selected this library size reasoning that at least half of the marketed drug molecules can be constructed from a limited set of privileged structures. In assembling the fragment set, we also took note of independent research findings on the origin of conserved metabolism, ligand–protein binding data from international structural genomics efforts, and scaffold framework diversification calculations for organic molecules.

To determine the utility of the FOL library, it was screened against leukotriene A4 hydrolase (LTA4H) by X-ray crystallography. LTA4H is a 69 kDa bifunctional enzyme with aminopeptidase and epoxide hydrolase activities that utilize a single zinc active site. The leukotrienes are a group of proinflammatory lipid mediators that have been implicated in the pathogenesis and progression of atherosclerosis. For example, it has been shown that a haplotype (HapK) spanning the LTA4H gene confers an increased risk of myocardial infarction, a finding that is consistent with a growing body of biochemical and cell biological research. Single nucleotide polymorphisms spanning the LTA4H gene have also been correlated with increased risk of asthma and allergy susceptibility. The importance of LTA4H as a therapeutic target is exemplified by the development of multiple inhibitors representing different chemotypes.

The active site pocket of LTA4H is a 17 Å long L-shaped cleft capable of binding the leukotriene A4 substrate. The crystal structure of LTA4H was originally solved bound to bestatin (I), a low molecular weight secondary metabolite of Streptomyces olivoreticuli and a component of the FOL library. The catalytic domain of LTA4H shares a high degree of structural similarity with the metalloprotease thermolysin including a conserved active site zinc binding motif (HEXXH-X18-E) (Figure 2A). Very little is known about the biological relevance of the LTA4H aminopeptidase activity. It is speculated that the enzyme may process peptides related to inflammation and host defense. As a hydrolase, the enzyme converts the oxirane ring of LTA4 to the respective dial, leukotriene B4 (LTB4), which acts as a chemoattractant and an activator of inflammatory responses mediated by binding to G-protein-coupled receptors BLT1 and BLT2.

In this report we show that the FOL library yielded fragment hits including natural molecules such as resveratrol and derivatives of nicotinamide. We also show how FOL hits could be elaborated into potent LTA4H inhibitors with drug-like properties representing novel chemotypes. These fragment-based inhibitors show good ligand efficiency for inhibition of peptidase and hydrolase activities in vitro.

### Results

**FOL Library Design.** In order to facilitate use of the FOL library in a diverse set of applications, each of its members was determined to have solubility in methanol at greater than 50 mM (see Materials and Methods). Individual members of the FOL library were maintained as 50 mM stocks in methanol which allowed them to be dispensed and dried into powders for subsequent dissolution into new formulations with no residual solvent (e.g., crystal soaking conditions). Within the FOL library, we defined natural molecules of life “FOL-Nat” to be any small molecule (MW ≤ 350) which is known to be produced by any living organism and therefore represented within the metabolome. Without bias for any particular metabolic pathway, the current FOL-Nat set includes 218 molecules of life. It is noteworthy that the first LTA4H crystal structure was obtained with bestatin I, a natural metabolite bound in the active site, and there are...
numerous similar examples of natural molecules leading to the design of drug candidates that modulate protein activity.\textsuperscript{52–54} Evolutionary concepts of substrate ambiguity and enzyme promiscuity suggest that many enzyme active sites have evolved flexibility to transiently bind substrates and products.\textsuperscript{55–58} We therefore decided to add heteroatom derivatives and isosteres of natural molecules of life to our fragment set. Termed “FOL-NatD”, these synthetic derivatives or isosteres of natural molecules of life are anticipated to be fortified with small modifications that could stabilize binding to enzyme active sites. The current FOL-NatD set includes 666 fragments. In order to emulate 3D protein architecture and to further diversify the FOL library, we have included a set of 445 synthetic biaryl molecules, many of
whose energy minimized structures mimic $\alpha$-, $\beta$-, and $\gamma$-turns. This subset of the FOL library was named “FOL-Biaryl.” All of the listed FOL concepts are summarized in Figure 1.

Our final 1329 member FOL library generally conforms to the “rule of three” guidelines for fragments (see Supporting Information Figure 1 for physical and chemical properties of the FOL library) and contains approximately 400 distinct chemotypes (see Supporting Information Figure 2 for calculated diversity properties of the FOL library). Members of the complete FOL library have an average molecular weight of 182.5, ClogP of 0.96, 1.49 hydrogen bond donors, 2.56
hydrogen bond acceptors, 1.7 rings, and 2.7 rotatable bonds. The only parameter that lies outside the rule of three is polar surface area with an average value of 118.9 Å³. This is reflective of the water-soluble properties of many small molecules of life. We reasoned that members of the FOL library represent “molecular rulers” providing information on the architecture and electronics of protein binding sites. Thus, our FOL library is designed to interrogate both enzyme active sites along with the more intriguing opportunities to probe allosteric pockets and protein–protein interaction surfaces.

**LTA4H Target FOL Screening.** To validate the FOL library as a screening set in a proof of concept study,
approximately 15% of the fragments were screened against LTA4H by soaking respective crystals with randomly selected pools each containing eight different structurally diverse FOL fragments (see details of Computational and Physical Pooling in Materials and Methods). The resulting crystals were frozen, complete X-ray diffraction data sets were collected, and the models were partially refined using an LTA4H protein-only model. When unaccounted electron density was observed, candidate fragments from the pool that were consistent with the shape of the density were soaked individually and additional complete data sets were collected. When definitive electron density from a single
compound soak was observed, the fragment was built into the electron density and the complete protein–fragment model was refined. Thirteen unique hits were identified from these experiments, 12 of which are summarized in Figure 2B–M, representing an overall hit rate of 6%. The 13th fragment, an FOL-Biaryl member, will be described in a separate publication. Crystallographic data collection and refinement statistics for all structures are listed in Supporting Information Table 1. LTA4H inhibition assay data for all compounds in this manuscript are listed in Supporting Information Table 2 and are also summarized in Figure 2.

Figure 2. Continued
Representative fragments from all categories of FOL were observed bound to LTA4H (FOL-Nat, FOL-NatD, and FOL-Biaryl). Nine of the 13 FOL hit compounds were discovered to bind independently within the L-shaped substrate binding cleft. The FOL-Biaryl compound 10 was found to bind on the surface of LTA4H, far from the active site. Compound 7 was simultaneously bound to both the surface and within the L-shaped cleft of LTA4H (see Supporting Information Figure 3, an image of LTA4H surface binding of 7). Three additional FOL-NatD molecules (11–13) were accommodated in the substrate binding cleft only upon concomitant binding of an additional inhibitor,
Flexible ligand. The IC$_{50}$ of and an entropic penalty to be paid by constraining a more because of an energetic trade-off between an improved fit did not translate to an increase in binding affinity, possibly resulting crystal structure showed very clear electron density synthesized 3.

Figure 2. Panels showing ligand binding to LTA4H for compounds described in the manuscript. Enzyme assay IC$_{50}$ values are in μM, human whole blood cell assay IC$_{50}$ values are in nM when reported, and ligand efficiency (LE) values are in kcal/(mol-heavy atom). Compound structures are displayed as yellow stick structures, and LTA4H is displayed in gray. Green mesh corresponds to the $F_o - F_c$ (difference) electron density at the 3.0σ level of the crystal structure with the compound omitted from the model. Polar contacts with LTA4H and/or bound water molecules are shown as red dashed lines. PDB IDs for each structure are indicated.

bestatin (I). The binding modes of individual fragments are briefly described below.

The natural product resveratrol (compound 2) is a plant-derived polyphenol, was identified as a hit in our screen (Figure 2B). This natural molecule has been implicated in cardioprotection and antiaging. The polyphenol nature of 2 suggests that it could make multiple hydrogen bonds with the target including conserved water molecules at the bottom of the cleft and the backbone carbonyl of Gln136. In our hands, 2 was a relatively weak binder of LTA4H with IC$_{50}$ values of 366 and 212 μM in the peptidase and hydrolase assays, respectively. The observed electron density for the molecule was of intermediate quality. The model of the bound fragment in the crystal structure refined with relatively high temperature factors. Analysis of the structure of the resveratrol complex further suggested that the bound compound exists in a strained conformation due to the rigid C7−C8 double bond. From this observation, we postulated that dihydroresveratrol (compound 3), a major metabolite of 2 observed in mammals, would have more rotational freedom and may bind more effectively to LTA4H. We synthesized 3 and soaked LTA4H protein-only crystals. The resulting crystal structure showed very clear electron density for 3, and the fully refined model exhibited lower relative temperature factors compared to 2 (Figure 2C). Specifically, bound 3 displayed a conformation inaccessible to 2, with a torsion angle of −161.2° around the C7−C8 bond (Figure 3A). The observed better fit of 3 for binding LTA4H did not translate to an increase in binding affinity, possibly because of an energetic trade-off between an improved fit and an entropic penalty to be paid by constraining a more flexible ligand. The IC$_{50}$ of 3 was comparable to that of the parent resveratrol 2: 145 μM in the peptidase assay and 247 μM in the hydrolase assay.

N-(Pyridin-3-ylmethyl)aniline 4 is classified as an FOL-NatD fragment. It shares structural similarities with nicotinamide, namely, a pyridin-3-carboxamide moiety. It was bound at the bottom of the substrate binding cleft of LTA4H near the resveratrol binding site (Figure 2D). Most interactions of 4 with the protein were hydrophobic except for a potential hydrogen bond between the aniline NH and the backbone carbonyl of Pro374. Compound 4 displayed IC$_{50}$ values of 1.32 and 1.67 mM in the peptidase and hydrolase assays, respectively. Notably, 4 displayed structural similarity compared to the phenoxymethylbenzene pharmacophore of the previously reported inhibitors of LTA4H (PDB IDs 3CHO, 3CHQ). Superposition of 3CHQ or 3CHO structures with our LTA4H-3 and LTA4H-4 complexes demonstrated that the corresponding atoms of the phenoxymethylbenzene were all within 0.7 Å of their counterparts in 3 or 4 (see Supporting Information Figure 4). The structurally related fragment 3-(phenoxymethoxy)pyridin-2-amine (5) identified in our screen featured a similar binding mode (Figure 2E). The 2-amino function of the pyridine ring is likely to mediate a potential 2.8 Å hydrogen bond with the backbone carbonyl of Pro374. This bond is shorter and has more favorable geometry compared to that between 4 and Pro374. Notably, the interaction between the amine and Pro374 moiety slightly perturbed the binding pose of the biaryl scaffold. Better binding interactions of 5 with the target translated into its better potency (IC$_{50}$ values of 30.8 and 619 μM in peptidase and hydrolase assays). It is noteworthy that the hydroxamic acid inhibitors of LTA4H, which also contain phenoxymethylbenzene, do not appear to occupy similar space in LTA4H, most likely because of the dominant interactions that the hydroxamic acid component has with the catalytic zinc ion (see Supporting Information Figure 4).

We categorized the pyridine analogue 6 as an FOL-NatD molecule. It features a pyridine ring present in several molecules of life including niacin, vitamin B3 (pyridine-3-carboxylic acid), and 4-pyridoxic acid, the catabolic breakdown product of vitamin B$_6$. In our studies, fragment 6 was bound to the hydrophobic center of the LTA4H cleft, in the same location as 4. The carbonyl oxygen of 6 was in a suboptimal position, namely, within 3.6 Å of the carbonyl oxygen of Trp311 (Figure 2F). This unfavorable interaction likely contributed to poor activity of the fragment in the biological assays (IC$_{50}$ = 3.7 and 5.3 mM in peptidase and hydrolase assays, respectively).
mediate between oxygen of Glu271. Finally, the binding site of conserved water molecules, as was observed for LTA4H. The thiophene ring did not displace the structurally conserved water molecules and the side chain of residue Phe362 are also color-coded to show the differences in the structures with and without bestatin. For crystallographic data, see Supporting Information Table 1.

Superposition of the structure of dihydroresveratrol (Figure 3). This molecule was also well situated to make polar contact with imidazole bound between Glu501 and Glu348. Imidazole is present in the crystallization buffer and is included in most LTA4H structures observed at medium or higher resolution.

Of all the fragments identified by crystallographic screening, only compound 10 (FOL-Biaryl) did not bind in the substrate binding cavity (Figure 2J). It interacted with the surface of the protein, in a pocket formed by Arg24, Ile188, and Asp182. We have further confirmed the LTA4H binding of 10 and 7 (which binds a different pocket on the surface of LTA4H) by both surface plasmon resonance and saturation transfer difference NMR studies (data not shown). Molecule 10 was not competitive with bestatin (1) an LTA4H competitive inhibitor (data not shown) and displayed little or no inhibitory activity against LTA4H (IC$_{50}$ > 1 mM). Therefore, the two shallow surface pockets mapped out by compounds 10 and 7 are unlikely to be useful targets for inhibition of LTA4H. However, our findings suggest that the application of FOL methodology may yield additional insight into the topology of surface domains or allosteric sites. It could be further used to develop modulators of protein–protein interactions.

Simultaneous LTA4H Binding of Two Fragments. The two most common approaches in FBDD are fragment evolution and fragment linking. The fragment evolution technique involves chemical optimization of the initial fragment hit to produce higher affinity ligands. Fragment linking is focused on assembling adjacent independently bound fragments into a single molecule to produce high affinity ligands. In order to expedite the transition from fragment hits to leadlike molecules, we explored the possibility of identifying dual simultaneous FOL hits in LTA4H. This “fragment plus fragment” approach was inspired by a growing number of cases where two small molecules bind within protein targets and contact each other. For example, this can be seen in the interaction of the plant hormone auxin (indole-3-acetic acid) and an inositol hexakisphosphate cofactor in the crystal structure of the TIR1-ASK1 complex.

In one “fragment plus fragment” approach, we soaked LTA4H crystals with FOL pools that were spiked with 1 mM bestatin (IC$_{50}$ = 178 nM in peptidase assay). Subsequent

Two additional FOL-NatD fragment hits (compounds 7 and 9, Figure 2G and Figure 2I) and two FOL-Biaryl (compounds 8 and 10, Figure 2H and Figure 2J) featured thiazole (a component of thiamine, vitamin B$_1$) or thiophene rings. Although several thiazole derivatives have been identified as promiscuous protein binders, this chemotype provided valuable structural insight into LTA4H. Specifically, molecules 7–9 displayed distinctly different binding modes to the enzyme. They mapped out space occupancy of the entire L-shaped substrate cavity of LTA4H (see Supporting Information Figure 5). For example, 7 was bound deep within the LTA4H cavity (Figure 2G). The aminothiazole group displaced two of the three structurally conserved water molecules at the bottom of the substrate binding cleft. The amino substituent of the thiazole group occupied a position roughly between binding sites of the two displaced water molecules and was within hydrogen bonding distance of the carbonyl oxygen of Phe362. Thiophene derivative 8 was bound at the other end of the LTA4H substrate binding cleft near the zinc binding site (Figure 2H). The primary amino group of the fragment was less than 4 Å from the zinc atom, 2.9 Å from Oε1 of Gln136, and 2.7 Å from a carboxyl oxygen of Glu271. Finally, the binding site of 9 was intermediate between 7 and 8, with the thiophene ring making hydrophobic contact with Phe314 and Leu369 (Figure 2I). The amino group of 9 did not make any hydrogen bonds with LTA4H. The thiophene ring did not displace the structurally conserved water molecules, as was observed for 8. Both 8 and 9 were relatively potent ligands of LTA4H with measured IC$_{50}$ values equal to or below 100 μM in both peptidase and hydrolase assays. Compound 7 afforded poor inhibitory activity with IC$_{50}$ values greater than 1 mM. This could be a result of its binding mode being deep within the substrate binding cleft, perhaps making it unable to interfere with substrate binding in either of our LTA4H enzyme assays. Interestingly, a second molecule of 7 appeared to be bound in the LTA4H–7 cocystal complex on the surface of the protein. Its phenol moiety was in position to make polar contact with the carboxyl side chain of Glu501 and the backbone carbonyl of Leu343 (see Supporting Information Figure 3). This molecule was also well situated to make polar contact with imidazole bound between Glu501 and Glu348. Imidazole is present in the crystallization buffer and is included in most LTA4H structures observed at medium or higher resolution.

Simultaneous LTA4H Binding of Two Fragments. The two most common approaches in FBDD are fragment evolution and fragment linking. The fragment evolution technique involves chemical optimization of the initial fragment hit to produce higher affinity ligands. Fragment linking is focused on assembling adjacent independently bound fragments into a single molecule to produce high affinity ligands. In order to expedite the transition from fragment hits to leadlike molecules, we explored the possibility of identifying dual simultaneous FOL hits in LTA4H. This “fragment plus fragment” approach was inspired by a growing number of cases where two small molecules bind within protein targets and contact each other. For example, this can be seen in the interaction of the plant hormone auxin (indole-3-acetic acid) and an inositol hexakisphosphate cofactor in the crystal structure of the TIR1-ASK1 complex.

In one “fragment plus fragment” approach, we soaked LTA4H crystals with FOL pools that were spiked with 1 mM bestatin (IC$_{50}$ = 178 nM in peptidase assay). Subsequent
screening of FOL yielded three different indole fragments (compounds 11–13) bound in the substrate binding cleft (Figure 2K–M). Notably, these fragments interacted with LTA4H only in the presence of bestatin. A representative example, 5-hydroxyindole (compound 11) was a component of the FOL-NatD subset. This core is exhibited in the 5-hydroxyindole-3-acetic acid structure, which is a serotonin metabolite25,26 (Figure 1). It was bound near residues Trp311 and Tyr378, with the hydroxyl oriented closest to the bestatin, 3.5 Å away (Figure 2K). Two halogenated analogues of 11, namely, 12 and 13 lined up with the target in a similar orientation. Both chlorine and fluorne atoms of 12 and 13, respectively, were ~5.7 Å away from the nearest atom of the phenyl ring of bestatin. The shift in the binding mode of these indoles likely results from their decreased polar surface area compared to 11. All obtained X-ray structures revealed well-defined electron density maps for bestatin with a binding mode essentially identical to that previously observed.26

In another approach, we sought to determine if the presence of bestatin could alter the binding mode of another fragment hit whose binding pose would be expected to be overlapping or incompatible with bestatin. Our rationale for this approach was to determine if the binding mode(s) of any fragments could be adjusted by induced-fit mechanisms resulting from the bestatin interaction with Zn$^{2+}$. With this approach, we found that bestatin could induce dihydroresveratrol (3) to adopt a different pose compared to the fragment 3 alone within the LTA4H substrate binding cavity (Figure 3A). Specifically, a brief LTA4H crystal soak of 3 (25 mM) followed by a 4 h soak with a mixture of 3 (25 mM) and bestatin (1 mM) resulted in a structure where 3 was displaced by ~2.5 Å from its original binding site. With bestatin chelating the Zn$^{2+}$ in its typical binding mode, molecule 3 was forced to bind at the bottom of the LTA4H substrate binding cleft. It displaced two of the three structurally conserved water molecules with its phenolic hydroxyl groups (Figure 3B) and was within a distance of ~3.3 Å to bestatin. The torsional angle about the C7–C8 bond featured by the molecule in a LTA4H–3–bestatin structure was approximately 169°. Resveratrol (compound 2) could not be visualized in a similar crystal cosoak with bestatin. We attributed this outcome to the lack of flexibility in 2 that is critical to the binding at the bottom of the binding cleft in the presence of bestatin. The discovery of a binding mode unattainable without a second fragment was an interesting outcome from the “fragment plus fragment” approach. In this case, bestatin altered the topology of LTA4H binding cavity and pushed dihydroresveratrol deeper into the cavity. As a result, the molecule adopted a novel binding mode requiring the side chain movement of Phe362. The new observed binding mode is intriguing, particularly in the displacement of structurally conserved waters. It is conceivable that fragment elaboration principles could be employed to produce potent inhibitor(s) that incorporates design concepts of both bestatin and the novel dihydroresveratrol interaction.

Acetate is one of the smallest of fragments, with just four non-hydrogen atoms and a molecular weight of 56. In LTA4H, acetate ions in the crystallization solution were often visualized binding to $\text{Yb}^{3+}$ ions that are located at a key crystal lattice interface.26,27,73 Similarly, we have observed acetate binding to the catalytic Zn$^{2+}$ in the high resolution crystal structures of LTA4H with FOL compound 9 (Figure 2I). We sought to extend the “fragment plus fragment” strategy by employing acetate bound at the catalytic zinc in combination with other fragments. Notably, when 200 mM acetate was included as an additive in an LTA4H crystal soak with compound 4, the final solved structure revealed both acetate and compound 4 binding simultaneously as anticipated from their previously observed individual binding modes. It is interesting to note that the acetate–Zn$^{2+}$ binding events together with the binding modes of compounds 4, 5, and 9 were essentially superimposable (see Supporting Information Figure 6). On the basis of this observation, we postulated that a proper linking of carboxylic acid functionality to a hydrophobic biaryl would yield a potent inhibitor. Of particular interest were templates that could provide for the proper dihedral angle and distances in positioning both biaryl and acetate pharmacophores within the active site of LTA4H. Physicochemical properties, good ligand efficiency, and chemical feasibility were additional criteria we considered. In the course of chemical studies, (R)-prolinol was selected as a tether between a hydrophobic biaryl and a metal-binding carboxylate, yielding DG-051 (19), a drug molecule that has completed phase Ia clinical studies.43 Thus, the DG-051 molecule (Figure 2S) displays two key pharmacophore features suggested by our fragment-based studies, namely, a carboxylic acid moiety binding to the active site zinc and a hydrophobic anchor at the bottom of the substrate binding cavity.

**Elaboration of FOL Fragment Hits.** The abundance of fragment screening data summarized above prompted us to improve the potency of several FOL hits through a limited medicinal chemistry effort. The rationale was to conceptually prove that the identified weak binders could be rapidly evolved into a potent LTA4H inhibitors following structural guidance obtained thus far. On the basis of the synthetic feasibility, we selected three fragments (4, 6, and 11) as templates for this chemical elaboration (see Schemes 1–3). In selecting proper pharmacophores for our chemistry effort, we considered both the trajectory for fragment linkage as suggested by the structural studies as well as druglike character of the designed molecules. Midsized cyclic amines were of particular interest, as they offered well-defined spatial orientation for the pharmacophores. Furthermore, these moieties were expected to mediate additional interaction within the active site and to enhance solubility and permeability of a fragment derivative across biological barriers. A brief synthetic effort followed by crystallographic screening converged on (R)-prolinol as a suitable tertiary amine tether. For example, considering the LTA4H binding mode of 4, we reasoned that amendment of 20 with the optimized (R)-prolinol linker of DG-051 would yield a more potent molecule, 14 (Scheme 1). Further, on the basis of the structural data for 7, wherein its aminothiazole was found to displace structurally conserved water molecules deep within the LTA4H active site cavity, we amended 23 to introduce both thiophene and (R)-prolinol groups to afford 18 (Scheme 1). Synthesis of the prolinol derived analogue 14 was accomplished by the nucleophilic displacement of tosyl group in 24 with the phenolate derived from 4-benzylaminophenol 20 (Scheme 1). This step was followed by the removal of Boc-protecting group from 25. The tosylate 24 was prepared following the literature procedure from (R)-Boc-prolinol. The Friedel–Crafts acylation of anisole with 4-iodobenzoyl chloride followed by reaction of the methyl ether 22 with boron tribromide in CH₂Cl₂ provided the phenol 23. The
sodium phenolate of 23, generated in situ with NaH in DMF, was subsequently reacted with the tosylate 24 to provide the BOC protected 4-iodobenzophenone derivative 26. Stille coupling of 26 with thiophene-3-boronic acid followed by deprotection of the N-BOC under acidic conditions provided the compound 18 (Scheme 1).

Subsequent structural studies of 14 revealed that the pyrrolidine moiety was bound at the bend in the LTA4H substrate binding cavity (Figure 2N). It provided the necessary kink for the molecule pivoting toward catalytic Zn\(^{2+}\). Its binding mode resembled the pyrrolidine group orientation in the DG-051-LTA4H complex (Figure 4), suggesting that a single carbon atom linkage between the two components would yield a molecule with LTA4H binding similar to that of DG-051 (Figure 2S). Structural studies of 18 (Figure 2R) confirmed that, similar to 7, the thiophene moiety displaced two water molecules from the bottom of the substrate binding cavity (Figure 5). The biaryl core of 18 was shifted compared to the parent molecule 6, moving carbonyl oxygens 5.2 Å apart. This motion likely decreased the unfavorable interaction with the backbone carbonyl of Trp311, resulting in a potent inhibitor with IC\(_{50}\) values of 80 nM in the peptidase assay and 189 nM in the hydrolase assay.

To further explore elaboration of fragment 6, we displaced the fluorine atom in 6 with N-pyrrolidin-2-ethanol to furnish

**Scheme 1. Synthetic Routes for New Molecules Inspired by the LTA4H Binding Modes of Compounds 4, 6, and 7\(^*\)**

**Scheme 2. Elaboration of Fragment 6\(^*\)**

**Scheme 3. Elaboration of Fragment 11\(^*\)**

\(^*\) Reagents and conditions: (a) AlCl\(_3\), 10 °C, nitrobenzene, 90%; (b), BBr\(_3\), CH\(_2\)Cl\(_2\), -78 °C → 0 °C, 85%; (c) NaH/DMF, 0 °C → rt → 90 °C; (d) 1 M HCl/Et\(_2\)O, 80–97%; (e) thiophene-3-boronic acid (2 equiv), Pd(OAc)\(_2\) (25 mol %), K\(_2\)CO\(_3\)/EtOH/DME, 90 °C, 60%.

\(^*\) Reagents and conditions used are as follows: (a) 1-(2-hydroxyethyl)pyrrolidine, K-O'Bu, DMSO, 90 °C, 16 h, 54%; (b) NaBH\(_4\), MeOH, 40 °C, 4 h.

\(^*\) Reagents and conditions: (a) 1-(2-chloroethyl)pyrrolidine, K\(_2\)CO\(_3\), acetone, 19%; (b) 1-bromo-2-chloroethane, K\(_2\)CO\(_3\), 2-butanone, reflux 60 h, 17%; (c) piperidine-4-carboxylic acid ethyl ester, HCl (2 equiv), KI (0.3 equiv), K\(_2\)CO\(_3\), DMF, rt → 90 °C, 57%; (d) aq NaOH, EtOH, rt.
with NaOH in EtOH to afford the targeted molecule ester derivative 16. Acid ethyl ester in refluxing 2-butanone furnished the primary chloro group of the 2-chloroethyl ether was first reacted with 1-bromo-2-chloroethane to provide 16. For the preparation of compound the carbonyl of Trp311 (Figure 2Q). Molecule hydrogen bond between the OH group in the molecule and an IC50 of 202μM in the peptidase and hydrolase assays, respectively, was 4-fold less than that of 15. Notably, the binding modes for molecules 15 and 16 significantly differed. Positioning of indole group (Figure 6) was driven by the orientation of pyrrolidine (for 15) and piperidine-4-carboxylic acid (for 16) pharmacophores in the active site of LTA4H.

Discussion

In this work we have combined the concepts of metabolo-mics with fragment based drug discovery to assemble a ~1300-member “fragments of life” (FOL) small molecule library for screening by X-ray crystallography. This set includes highly soluble metabolites, natural products, their derivatives, and biaryl molecules mimicking architectural motifs found in proteins. Small molecules of life have coe-volved with their respective macromolecular targets, and therefore, such interactions are likely to be highly ligand efficient. In addition, signaling pathways are known to display allosteric feedback mechanisms involving small molecules (for a recent review, see Goodey and Benkovic[84]). Consequently, application of molecules of life in the FBDD approach may lead to the identification of novel allosteric binding sites in protein targets. In addition, many molecules of life are likely to feature druglike properties, since these compounds display properties necessary for intracellular activity (e.g., reasonable target affinity, reversible binding, controlled on/off rates, solubility, permeability, etc.).

The FOL library has been designed without target bias. In the initial validation, we screened approximately 200 molecules (15%) of the entire selection (1329 compounds) against LTA4H using X-ray crystallography. Thirteen unique
fragments were discovered, representing a hit rate of 6%. Most of the identified fragments (12 out of 13) were bound to the active site pocket, while fragment 10 bound within a shallow surface pocket. Fragment 7 interacted with both the active site pocket and the surface of LTA4H. It is noteworthy that the two fragments found binding to the surface of LTA4H were biaryl structures with either a thiophene or thiazole moiety plus at least one amino or hydroxyl group. A similar observation has been reported recently for thiazole derivatives binding at sites of protein–protein interaction, suggesting the possibility that these themes may be exploited to develop specialized fragment libraries to screen for modulators of protein–protein interactions. Importantly, FOL hits varied both structurally and in their source within the library. Specifically, biological molecules FOL-Nat (e.g., resveratrol, dihydroresveratrol, 5-hydroxindole), their derivatives FOL-NatD (e.g., 5-hydroxyindole, nicotinamide, and isonicotinic acid derivatives), and FOL-Biaryls which mimic a protein structure (e.g., fragment 10) were found to bind to LTA4H in a diverse array of binding modes.

The structures of LTA4H with bound resveratrol (compound 2) and its metabolite dihydroresveratrol of were of particular interest because of the potential therapeutic implications. As LTA4H is involved in both increased risk of myocardial infarction and inflammation, one may consider attributing the apparent cardioprotective properties of resveratrol and other sirtuin activators to LTA4H modulation. Our finding suggests that the FOL approach may

Figure 5. Elaboration of 6. (A) Superposition of compounds 6 (yellow), 17 (green), and 18 (cyan). Invariant portions of the LTA4H structure are colored gray, and compound-specific polar contacts, side chain conformations, and bound water molecules are color-coded by compound. (B) Superposition of 18 (cyan) with the thiazole-containing fragment 7 (rose) and the pyrrolidine containing compound 14 (magenta). Both 7 and 18 displace the central structurally conserved water molecule, and both 14 and 18 have pyrrolidine moieties that occupy nearly the same space in the LTA4H structures.

Figure 6. Elaboration of 5-hydroxyindole 11, showing superposition of the indole-containing fragment structures: 5-hydroxyindole 11 (dark green), 5-chlororindole 12 (magenta), 5-fluoroindole 13 (cyan), and derivative compounds 15 (yellow) and 16 (pink).
facilitate rational identification of small molecule agents that simultaneously modulate several therapeutically relevant pathways.\(^8\)

The binding of dihydroresveratrol 3 also illustrates the potential utility of “fragment plus fragment” mode of screening. For example, when 3 bound to LTA4H together with bestatin, several structural water molecules were displaced within the LTA4H active site pocket and Phe362 was found to adopt an alternative side chain rotamer conformation. This finding shows that when fragment cocktails are repooled with other fragments, new induced-fit fragment binding modes can be observed.

Using structural guidance from FOL screening, we showed that fragment hits could be expeditiously modified to generate potent LTA4H inhibitors in a short synthetic sequence. The diverse chemical nature of the initial hits suggests that this strategy is applicable to the identification of novel and structurally distinct chemotypes. In a representative example, structural information from diverse active fragments was combined to evolve a fragment hit 6 of very poor activity (IC\(_{50}\) = 5.3 mM) in the peptidase assay into a potent LTA4H inhibitor 18 (IC\(_{50}\) = 80 nM) in two synthetic steps. Fragment hit 4 was derivatized in a single step to afford 14, a novel and potent inhibitor of LTA4H (IC\(_{50}\) = 150 nM) in the hydrolase assay with comparable potency (IC\(_{50}\) = 131 nM) for inhibition of LTB4 production in human whole blood. Compound 14 featured high ligand efficiency of 0.44 kcal/(mol·heavy atom) vs 0.36 kcal/(mol·heavy atom) for the clinical candidate DG-051 19. Thus, a combination of properly selected fragments with structural insight into their mode of binding allowed for maintenance of ligand efficiency and druglike potential of the optimized hits.

The FOL library was of value in expanding the known chemical space of LTA4H inhibitors. Our structural studies have consistently identified multiple fragments that exemplify reported LTA4H binders. For example, the binding modes of our compounds 4 and 5 were all superimposed and mimicked the benzoxypyphenyl components of LTA4H inhibitors reported by Kirkland et al. (see Supporting Information Figure 4).\(^40\) Furthermore, our ligand docking studies suggest that the predicted binding modes of the LTA4H inhibitors reported by Grice et al. and Rao et al.\(^39,88\) could be emulated with FOL hits. In a representative example, structural information from diverse active fragments was also captured binding trajectories for multiple reported inhibitors of the enzyme. We further demonstrated that the identified fragments could be optimized in a short reaction sequence to yield molecules with high ligand efficiency and potent biological activity. The fragments and their derivatives feature binding efficiency for LTA4H and surface efficiency indices that match those of marketed drugs\(^36\) (see Supporting Information Figure 9). Further studies are required to determine the versatility of the FOL library screening against diverse target classes. It is likely that further chemical amendments to this selection will be needed to enhance hit rates. In addition, the versatility of the FOL set is being tested in different readout platforms including NMR and surface plasmon resonance.

Materials and Methods

Purification of LTA4H. Recombinant human LTA4H (residues 1–611) was expressed in *E. coli* BL21-AI/pRARE. An amount of 7.5 g of cell paste was lysed by nitrogen cavitation at 2200 psi for 1 h in a lysis buffer consisting of 50 mM Tris-HCl, pH 8.0, 1 mM PMSF, 0.2 mg/mL lysozyme, and 1.5 U/mL benzonase. The lysate was clarified by centrifugation at 42 000 rpm for 45 min at 4 °C. Clarified lysate was filtered using a 0.2 μM syringe-top filter. Lysate was then applied to 2 × 5 mL HisTrap HP columns (GE Healthcare) equilibrated with 20 mM Tris-HCl, pH 8.0, and 200 mM NaCl. The column was washed with six column volumes of 30 mM imidazole and eluted with a linear gradient of 30–300 mM imidazole in 20 mM Tris-HCl, pH 8.0, containing 200 mM NaCl. The pooled peak fractions were >95% pure by SDS–PAGE at a concentration of 2.7 mg/mL and used directly for crystallization without further concentration or dialysis.

Crystallization and Fragment Screening. LTA4H was crystallized by sitting drop vapor diffusion against a crystallant containing 100 mM imidazole, pH 6.5, 12.67% w/v PEG-8000, 100 mM sodium acetate, and 5 mM YbCl\(_3\). Crystal growth was accelerated through the use of a microseeding protocol. Seed stocks were prepared by suspending several crystals in 20 μL of crystallant and crushing them by vortexing in a Seed Bead tube (Hampton Research). Crushed crystals were diluted 1:100 into fresh crystallant and vortexed again to form the final seed stock. Crystallization drops were set up in a Compact Jr. 96-well crystallization plate (Emerald BioSystems) by combining 0.7 μL of LTA4H (2.7 mg/mL) with 0.7 μL of crystallant and 0.2 μL of seed stock over a reservoir volume of 100 μL.

X-ray Crystallography. Twenty-five pools of eight compounds from the FOL library were screened against LTA4H by X-ray crystallography. Pool stocks consisted of cocktails of eight fragments, each at 6.25 mM in methanol. Individual FOL compounds are stored in barcode labeled sealed septum vials as
50 mM stocks in pure methanol and stored at −20 °C. Approximately 30% of the fragment stocks have been analyzed over a period of 12 months by mass spectrometry to demonstrate general stability of the compounds in methanol (data not shown). Fragment cocktails in methanol were spotted onto the drop chambers of crystallization plates and the solvent was allowed to evaporate, leaving a precise amount of fragment compounds as a dry powder with no solvent residue that could potentially interfere with crystallization or crystal stability. A volume of crystallization mother liquor equal to the initial volume of fragment cocktail was added to the dry powder, allowing for dissolution of the fragments. Crystals were transferred to the resulting solution and allowed to soak overnight. This technique is versatile, as it is suitable for both co-crystallization and soaking experiments, and adjusting ratios of methanol solution to crystallant solution allows for adjustment of the final fragment concentration in the drop.

Although diversity pooling was conducted to aid in identification of fragments from pool density alone, factors such as low resolution, low occupancy, or competitive binding can lead to uncertainty. Therefore, in cases where crystals exposed to fragment cocktails produce difference electron density indicative of fragment binding, follow-up experiments with individual fragments were performed. During this deconvolution step, individual candidate fragments were soaked into crystals, usually at 10−25 mM. The resulting crystal structures provide unambiguous confirmation of fragment hits, and all structures presented were generated from single molecule crystal soaking experiments.

**LTA4H Peptidase Activity Assay.** L-Arginine-p-nitroanilide (Arg-pNA) substrate was obtained from Sigma. Stock solution of Arg-pNA (500 mM) was prepared and serially diluted in DMSO. Final peptidase assays were performed in 96-well clear, flat bottom plates (Corning). Each assay plate contained eight positive (no inhibitor) and eight negative (no enzyme) controls. LTA4H (45 μL) and test compounds (5 μM to 1 μM) were preincubated for 10 min followed by addition of substrate (50 μL); final reactions contained 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5% DMSO, 0.5 mM Arg-pNA, and 20 nM LTA4H. Control experiments showed that 5% DMSO had no effect by itself on enzyme activity (data not shown). Reaction rates were monitored in a SpectraMax 190 plate reader ( Molecular Devices) at room temperature (r.t) by measuring the increase in absorbance at 405 nm change to cleavage of the methanol p-nitroanilide. Reactions were measured for 30 min, and initial rates were determined from the linear portions of the progression curves using SoftMax Pro software ( Molecular Devices). Dose−response curves were calculated by fitting data to a sigmoidal four-parameter logistic equation using Prism (Graphpad).

**LTA4H Hydrolase Activity Assay.** LTA4 substrate was prepared from the methyl ester of LTA4 (BioMol or Cayman Chemicals) by treatment under nitrogen with 100 mol equiv of NaOH in an acetone/H2O (4:1) solution at r.t for 40 min. Stock solutions of LTA4 were kept frozen at −80 °C for a maximum of 1 week prior to use. Recombinant human LTA4H (10 nM final concentration) was incubated with various concentrations of test compound for 10 min at r.t in assay buffer (0.1 M Tris-HCl, 0.1 M NaCl, 5 mg/mL fatty acid free BSA, 10% DMSO, pH 8.0). Immediately before the assay, LTA4 was diluted in assay buffer (without DMSO) and added to the reaction mixture to a final concentration of 1 μM to initiate the enzyme reaction. Samples were incubated for 10 min at r.t followed by addition of 2 volumes of chilled quenching buffer (CH3CN with 1% CH3COOH and 73 mM LTB4-d4 (BioMol). The samples were then stored at 4 °C for 12 h to complete protein precipitation and centrifuged for 15 min at 1800g. LTB4 formed was measured in the supernatant by LC/MS/MS using LTB4-d4 as an internal standard and an external LTB4 standard (BioMol) for a calibration curve. Briefly, the analyte was separated from LTB4 isomers formed by spontaneous hydrolysis of LTA4 using isotropic elution on an HPLC system (Waters) and analyzed on a tandem quadrupole mass spectrometer (Waters Micromass Quattro Ultima). MRM transitions for LTA4 and LTB4-d4 were 335.2→195.3 (LTB4) and 339.2→197.3 (LTB4-d4). On the basis of the measured amounts of LTB4 formed at each inhibitor concentration, a dose−response curve was fitted to the data using a sigmoidal four-parameter function (Prism, Graphpad) and an IC50 value was calculated. Ligand efficiency values were calculated for hydrolyse and peptidase assays independently using LE (kcal/(mol·HA)) = −RT(ln(IC50)HA) = −0.59179−(ln IC50)/HA, where HA is the number of heavy atoms (non-hydrogen).

**Human Whole Blood LTB4 Assay.** Human blood (45 mL) was collected in heparin-containing Vacutainer tubes (Greiner-Bio One) with informed consent. Individual experiments were performed with blood from a single subject. For each sample, 200 μL of blood was dispensed into a prewarmed 96-well plate and 188 μL of RPMI-1640 medium (Invitrogen) containing 20 μg/mL indomethacin (Sigma) was added. An amount of 4 μL of a series of compound dilutions (final DMSO concentration of 1%) was added in triplicate, followed by a 15 min incubation at 37 °C with gentle shaking. Blood samples were stimulated by adding 8 μL of ionomycin (from Streptomyces conglobatus, Calbiochem) to a final concentration of 36 μM. After another incubation at 37 °C for 30 min, samples were centrifuged at 4 °C for 5 min at 1800g. LTB4 concentrations in supernatants were determined using a commercially available enzyme-linked immunosorbent assay (R&D Systems) according to the manufacturer’s instructions. On the basis of the measured amounts of LTB4 formed at each inhibitor concentration, a dose−response curve was fitted to the data using a sigmoidal three-parameter function (XLfit, IDBS), and an IC50 value was calculated. Each assay plate contained eight positive controls (no compound) and eight negative controls (no Ionomycin).

**Fragment Selection for the Fragments of Life Library.** Approximately 400 candidate fragments representing natural molecules of life with (i) molecular weights less than 350, (ii) fewer than six hydrogen bond donors, and (iii) fewer than seven hydrogen bond acceptors were manually selected from known metabolic pathways. Molecules were selected from eukaryotic, archaeabacterial, and eubacterial metabolic pathways and from presumed primordial pathways.89,90 There was no bias toward any particular metabolic pathway; however, conserved intermediary metabolism were prioritized.91 Approximately 300 of the resulting fragments were commercially available, reasonably priced, and acquired. These fragments were tested for solubility (≥50 mM in methanol) to yield a final core set of 218 natural molecules (FOL-Nat). The chemical diversity of the first 218 FOL-Nat fragments was determined using a 210×210 principal component analysis (PCA) using Cerius2 (Accelrys). In this analysis 1D, 2D, and 3D descriptors (conformational, cat shape, electronic, quantum chemical, information content, spatial, structural, thermodynamical, topological, and geometrical) were calculated and the 3D distribution of the fragments was visualized in a 3D plot (see Supporting Information Figure 2). This analysis showed that the fragments were diverse but did not densely cover the desired chemical space. Therefore, in addition to the manual selection, we explored the Available Chemicals Directory (ACD/ Lab, version 7, MDL) and the Maybridge Fragment Library (www.maybridge.com) as a source of additional fragments. We intended to diversify the original set by selecting heteroatom-containing derivatives of natural molecules (FOL-NatD) with fragment-like properties. Toward this goal, a selection of 238996 compounds were analyzed using modified “rule of three”11 parameters to afford 49 877 fragments with (i) molecular weights between 110 and 250 Da, (ii) calculated log of octanol/water partition coefficient (ClogP) of less than or equal to 3.0 (calculated with software available from Daylight
Chemical Information Systems, Inc.), (iii) hydrogen bond donors less than or equal to 3, and (iv) hydrogen bond acceptors less than or equal to 6 (calculated with Sybyl, version 6.8, Tripos Inc., St. Louis, MO). The selection of the 4157 molecules, subjected to a panel of SMARTS filters\(^5\) in order to eliminate molecules endowed with problematic functionalities. A partial list of these SMARTS filters included reactive functional groups, lipophilic chains of seven or more carbon atoms, crown ethers, disulfides, excessive (\(>3\)) acidic groups, thiols, epoxides, aziridines, hydrazones, thioureas, thioyanates, benzylic quaternary nitrogens, thioesters cyanamid, four membered lactones, di- and triphosphates, metals, phosphines, phosphonic acids, sulfonic acids, sulfonil halides, boronic acids, isotopes, salts, metals, more than 3 halogens, more than two nitro groups, and lanthanides. This culling process resulted in 40 489 candidate fragment compounds. Each fragment from the resultant filtering sequence was converted from 2D to 3D projection using CONCORD (Tripos) and used to identify fragments with \(\leq 3\) rotatable bonds and calculated PSA values of \(\leq 60\,\text{Å}^2\). The resulting 5606 fragments were again analyzed using the previously described principal component analysis (PCA), and 1016 unique fragments were selected with preference for molecules that could be visually identified to have at least 8 atoms with chemical arrangements that matched that of a known natural molecule of life. From this set, 880 fragments were commercially available and obtained at a reasonable price. These fragments were tested for solubility (\(>50\,\text{mM}\) in methanol), and the resulting 666 fragments (FOL-NatD) were added to the FOL library. PCA analysis showed that the FOL-NatD fragments occupy distinctly different chemical space (see Supporting Information Figure 2) and together the FOL-Nat and FOL-NatD fragments combine to form a diverse and relatively dense set of fragments. Finally, we were also intrigued by the possibility of mimicking a protein 3D architecture using biaryl small molecule fragments.\(^9^6\) In order to identify protein mimetic fragments (FOL-Biaryl), we first identified 566 molecules containing 5–5, 6–5, and 6–6 biaryls connected via a \(\alpha\)-bond to allow for a controlled torsional freedom from the ChemBlock library (www.chemblock.com). The energy minimized conformations (Tripos force field, Sybyl 6.8) of a selection of these biaryl fragments were overlaid with the \(\alpha, \beta, \gamma\) turns of a known protein structure (1RTF.pdb) and shown to have good steric and electronic mimicry of protein structure. The resulting fragments were tested for solubility (\(>50\,\text{mM}\) in methanol), resulting in 445 final molecules (FOL-Biaryl). Results from the PCA analysis and physical properties of the fragments [MW, CloP, total polar surface area, number of hydrogen bond donor/acceptors, rotatable bonds, and rings] of the complete 1329 member FOL library are provided in Supporting Information (see Supporting Information Figure 1).

**Computational and Physical Pooling.** When fragments are pooled into structurally diverse cocktails of \(\approx 10\) compounds per mix, the throughput of X-ray screening of fragment libraries can be significantly increased.\(^13,94,95\) The pooling of fragments was carried out using the same \(210 \times 210\) descriptor space principle component analysis (Cerus\(^2\), Accelrys) in order to select eight diverse fragments per pool. The nature of the fragment (FOL-Nat, FOL-NatD, FOL-Biaryl) was not considered in the pooling strategy. Monte Carlo optimization using “Diversity Metric Function MAXMIN Distance” (Accelrys) with the optimum distance range of 1.97–2.57 Å was used to randomly extract eight diverse fragments. After extraction of eight fragments from the library, the process was repeated until the entire library was computationally pooled into structurally diverse pools each containing eight fragments. Individual fragments, each \(>95\%\) pure as determined by NMR spectroscopy, were prepared as 50 mM stocks in methanol. Physical pools of eight fragments each were prepared by mixing fragment stocks such that the final concentration of each was \(\sim 6\,\text{mM}\). The fragment pools were found to be visually stable, with no observed precipitation, suggesting that fragment pairs or combinations do not have a propensity to aggregate.

**Molecular Docking.** The ligand docking into LTA4H was carried out with the Xray molecules fragmented in Sybyl 7.2 (Tripos Inc., St. Louis, MO). The Sulfrel-Dock program uses an empirical scoring function and a patented search engine to dock ligands into a protein’s binding site.\(^96\) The PDB ID 3CHI file was converted to a mole2 file, and hydrogen atoms were added. The \(B\)-values were replaced by the AMBER charges using Sybyl 7.2. The bound ligand of 3CHI was removed from the binding site. The Sulfrel-Dock protocol, a computational representation of the intended binding site, was generated using the probes \(\text{CH}_2, \text{N}^{-}\text{H}, \text{and } \text{C}=\text{O}\). The protocol directed the placement of the ligand during the docking process. LTA4H inhibitors were drawn in ISIS Draw and converted to 3D structures with CONCORD of Sybyl 7.2 (Tripos Inc., St. Louis, MO). All of the inhibitors were properly typed with hydrogens, and a 3D structure data file was created for docking. Each ligand was then fragmented which reduced the conformational space to be explored. The fragments were then aligned to the protomol probes. All the fragments were scored,\(^97\) and the highest scoring fragment was kept as the head fragment. The tail fragments were selected on the basis of the similar principle, and gradual attachment was carried out to build the docked ligand. The poses were refined, eliminating those with excessive penetration into the protein. Thirty poses for each of the ligand were generated and visualized in the active site based on their Sulfrel scores. The best fitting ligand poses were chosen for further comparative structural analysis.

**General Methods.** All reagents and anhydrous solvents were obtained from commercial sources and used without further purification unless otherwise noted. NMR spectra were recorded at 400 or 500 MHz (Varian Instruments) in the solvent indicated, and TMS was used as an internal reference. AC- DLabs NMR software was used to process FIDs to generate spectral parameters (\(\delta\) (ppm)/Hz). Coupling constants (\(J\)) are given in Hz. Mass spectra were obtained using either APCl or electrospray ionization (PE-SCIEX single-quad or Agilent mixed-mode units). Elemental analyses were carried out by Galbraith Laboratories, Inc. (Knoxville, TN) or Midwest Microlab, LLC (Indianapolis, IN). Column chromatography was carried out with the solvents indicated with silica gel (MP EcoChrom, 32-63D, 60 Å). The HPLC method was as follows. Compounds were eluted using a gradient of 90:10 to 10:90 A/B over 40 min at a flow rate of 0.5 mL/min, where solvent A was 0.05% TFA in 100% H₂O and solvent B was 0.05% TFA in 100% acetonitrile. For HPLC data (final products), peak area percent and retention time (\(t_R\) in min) are provided. The following compounds were obtained from commercial sources: compound 4 from Matrix, compounds 5, 7, 8, 9, and 10 from Maybridge, compound 6 from VWR, and compounds 11, 12, and 13 from Aldrich.

**Benzyl[4-((R)-1-pyrrolidin-2-ylmethoxy)phenyl]amine (14).** To a solution of 25 (148 mg, 0.387 mmol) in methanol (3 mL) was added HCl (1 M in diethyl ether, 6 mL). The mixture was stirred at rt for 3 h. The solvent was removed in vacuo to provide the title compound 14 as a hydrochloride salt (128 mg, 93%). \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta 1.60–1.73\) (m, \(1\,H\)), 1.87–2.12 (m, \(3\,H\)), 3.17–3.21 (m, \(2\,H\)), 3.84–3.87 (m, \(2\,H\)), 4.19–4.23 (dd, \(1\,H\)), \(J_1=3.6\,Hz, J_2=10.8\,Hz\), 4.11–4.16 (m, \(1\,H\)), 4.44 (s, \(2\,H\)), 7.0 (d, \(2\,H\) \(J=8.8\,Hz\)), 7.29–7.38 (m, \(5\,H\)), 7.48–7.49 (m, \(2\,H\)) 9.1 (s, \(1\,H\)), 9.8 (s, \(1\,H\)). LCMS: 97%; APCl \(m/z\) 283 (M+1). Anal. (C\(_{25}\)H\(_{23}\)N\(_2\)O-2HCl) C, H, N.

**5-(2-Pyridyl-1-ylethoxy)-lH-indole (15).** To a solution of 5-hydroxyindole 11 (446 mg, 3.35 mmol) in anhydrous aceton (20 mL) was added potassium carbonate (1.36 g, 9.8 mmol) and 1-(2-chloroethyl)pyrroline hydrochloride (1.28 g, 7.5 mmol). The reaction was heated to reflux for 16 h. After the mixture was cooled to rt, a solution of tetrabutylammonium bromide (200 mg, 0.6 mmol) in DMF (10 mL) was added and the mixture
was heated to 55 °C for 16 h. The solvent was removed in vacuo, and the resulting residue was partitioned between EtOAc (25 mL) and water (50 mL). The organic layer was separated, dried with anhydrous MgSO₄, and concentrated in vacuo to an oil. The oil was purified by silica gel flash chromatography (~100 g of SiO₂, 0–5% MeOH/CH₂Cl₂, gradient) to provide the title compound 15 (145 mg, 19%). ¹H NMR (400 MHz, DMSO-d₆) δ 1.68 (dt, J = 6.57, 3.15 Hz, 4 H), 2.53 (br s, 4 H), 2.78 (t, J = 6.04 Hz, 2 H), 4.03 (t, J = 6.04 Hz, 2 H), 6.31 (br s, 1 H), 6.71 (dd, J = 8.72, 4.2 Hz, 1 H), 7.03 (d, J = 2.15 Hz, 1 H), 7.25–7.29 (m, 2 H), 10.88 (br s, 1 H). LCMS (APCI⁺): m/z found 289 (M + H).

4-Iodophenyl(4-methoxyphenyl)methane (22). Nitrobenzene (45 mL) was cooled in an ice bath and treated portionwise with AlCl₃ (13.5 g, 101 mmol, 1.15 equiv) and followed by addition of 4-iodobenzoic acid chloride 21 (25 g, 94 mmol, 1.07 equiv) in nitrobenzene (25 mL) while maintaining a maximum of 10 °C. The reaction mixture was stirred at 0 °C for 10 min, whereupon aniseole (9.5 g, 88 mmol, 1 equiv) was added dropwise in such a manner that the temperature didn’t exceed 10 °C. The solution was left to warm to rt overnight. The yellow suspension was poured into ice-water (750 mL). The precipitate was collected by filtration and washed with water (3 × 25 mL). Residue was dissolved in CH₂Cl₂ (1 L), washed sequentially with aqueous NaHCO₃ (2 × 150 mL), dried over anhydrous MgSO₄, and concentrated in vacuo to provide the title product 22 (26.7 g, 90%). ¹H NMR (400 MHz, CDCl₃) δ 3.89 (s, 3 H), 6.96 (d, J = 8.4 Hz, 2 H), 7.48 (d, J = 8.0 Hz, 2 H), 7.79 (d, J = 8.4 Hz, 2 H), 7.84 (d, J = 8.0 Hz, 2 H). MS (APCI⁺): m/z calculated for C₁₄H₁₈O₂: 238.2; m/z found 239.3 (M + 1).
23 (324 mg, 1 mmol). The mixture was allowed to warm to rt and was stirred for 30 min. The mixture was again cooled to 0 °C, and 24 (400 mg, 1.1 mmol) in 1 mL DMF was added. The resulting mixture was allowed to warm to rt, stirred for 30 min, and heated to 95 °C for 16 h. The reaction was cooled to rt and poured into ice-water (100 mL), and the mixture was stirred for 30 min. The resulting solid was filtered, and the filtrate was concentrated in vacuo. The resulting potassium carbonate (5.5 g, 40 mmol) and 1-bromo-2-chloro-11-xyindole were added. The resulting mixture was added to water to rt and then heated to 90 °C for 16 h. The reaction mixture was poured into ice-water (100 mL) and extracted with 3 × 25 mL of EtOAc. The organic layer was washed with water (50 mL) and brine (50 mL), then dried over anhydrous MgSO4 and concentrated in vacuo. The crude mixture was purified by flash chromatography (∼150 g of SiO2, 0–7.5% 7 ANH N2H1; 1.20 MeOH/CH2Cl2 mixture, gradient) to provide the title compound 27 (1.2 g, 54%). 1H NMR (400 MHz, CDCl3) δ 1.89 (d, J = 6.48 Hz, 2 H), 6.48 (br s, 1 H), 6.88 (dd, J = 8.8, 2.3 Hz, 1 H), 7.12 (d, J = 2.0 Hz, 1 H), 7.19 (t, J = 2.7 Hz, 1 H), 7.30 (d, J = 1.0 Hz, 1 H), 8.07 (br. s, 1 H).

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Supporting Information Available: Nine figures showing property distribution for the FOL library; 3D diversity plot of the FOL library components and the LTA4H hits; binding mode of compound 7 to the active surface of LTA4H; comparison of LTA4H binding by dihydroresveratrol (3) to compound 4, benzoxyphenyls (from PDB codes 3CHQ and 3CHO), and a hydroxamic acid (docked to match the reported structures of Thunnissen et al.; triphene and thiazole ring containing fragments (7–9) binding into LTA4H L-shaped pocket; acetate coordinating active site Zn2+ together with fragments bound; indole structures providing coverage of the benzoazole and benzthiazoles of Grice et al. and Rao et al.; superposition of LTA4H binding modes of fragments 3–6 compared to Kirkland et al.’s 3CHO and 3CHQ, with docked poses for Grice et al.’s 10a and 10e and Rao et al.’s 26993135; BEI vs SEI plots for fragments and their derivatives; and two tables listing X-ray crystallographic data and refinement statistics and comparative IC50 data for peptidase, hydrolase, and HBW assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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