

# Molecular Docking and Pharmacophore Filtering in the Discovery of Dual-Inhibitors for Human Leukotriene A4 Hydrolase and Leukotriene C4 Synthase

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Combination of drugs for multiple targets has been a standard treatment in treating various diseases. A single chemical entity that acts upon multiple targets is emerging nowadays because of their predictable pharmacokinetic and pharmacodynamic properties. We have employed a computer-aided methodology combining molecular docking and pharmacophore filtering to identify chemical compounds that can simultaneously inhibit the human leukotriene hydrolase (hLTA4H) and the human leukotriene C4 synthase (hLTC4S) enzymes. These enzymes are the members of arachidonic acid pathway and act upon the same substrate, LTA4, producing different inflammatory products. A huge set of 4966 druglike compounds from the Maybridge database were docked into the active site of hLTA4H using the GOLD program. Common feature pharmacophore models were developed from the known inhibitors of both the targets using Accelrys Discovery Studio 2.5. The hits from the hLTA4H docking were filtered to match the chemical features of both the pharmacophore models. The compounds that resulted from the pharmacophore filtering were docked into the active site of hLTC4S and the hits those bind well at both the active sites and matched the pharmacophore models were identified as possible dual inhibitors for hLTA4H and hLTC4S enzymes. Reverse validation was performed to ensure the results of the study.

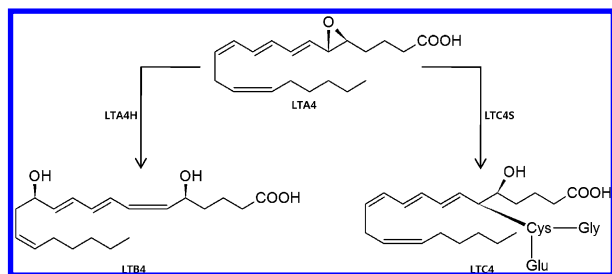
## INTRODUCTION

Disorders such as cancers, cardiovascular diseases, dementias, depression, and many others basically result from multiple molecular abnormalities, not from a single defect. In addition, systems biology has revealed that human cells and tissues are composed of complex, networked systems with redundant, convergent, and divergent signaling pathways.<sup>1</sup> Most of the biological pathways and networks frequently find ways to repair or counterbalance the protein which is targeted by a particular drug. It is highly realized recently that modulation of multiple targets would greatly benefit the treatment of diseases such as cancer and atherosclerosis.<sup>2</sup> This well balanced modulation of multiple targets has been quite successful in various therapeutic areas such as a dual acting anti-inflammatory drug ML3000 [2,2-dimethyl-6-(4-chlorophenyl)-7-phenyl-2,3-dihydro-*H*-pyrrolizine-5-yl] acetic acid, a dual inhibitor of 5-lipoxygenase (5-LOX) as well as both cyclooxygenases (COX-1 and COX-2) and another dual angiotensin-converting enzyme and neutral endopeptidase inhibitor, omapatrilat.<sup>3</sup> Whereas ML3000 has not yet been approved for use, omapatrilat failed in phase III clinical trials because of a mechanism-based adverse effect. Thus, searching for multitarget drugs emerges as an active area of research. Multitarget therapeutic strategy can be achieved by one of the following ways: (i) acting upon different targets to create a combination effect (e.g., Bactrim, which acts on two targets in the folate biosynthesis pathway

in bacteria), (ii) altering the ability of another to reach the target, and (iii) binding the different sites on the same target to create a combination effect.<sup>4</sup> Before the advent of multitarget strategy, combinations of different inhibitors that target a specific single target were the mode of treatment for diseases in which the drug–drug interaction is the major disadvantage. Multitarget strategy has become so advantageous over combination drug therapy since the better pharmacokinetic and pharmacodynamic profile was achieved as a result of administration of a single drug rather than combination of two drugs.<sup>3,5–7</sup> Multitarget drugs can be more effective and less vulnerable to adaptive resistance due to the smaller ability of the biological system to compensate the multiple actions exerted simultaneously.<sup>4</sup> A number of multitarget drugs that are in clinical use are the unexpected outcome of various drug discovery studies. Most of the reported combination strategies were inefficient because the combined ligands contain groups important to one target and not allowed by other targets.<sup>8,9</sup> Multitarget therapeutics are mostly developed as a mixture of agents with selectivity for individual targets and led to larger and complex ligands with poor pharmacokinetic properties. Thus, the major challenge in designing multitarget drugs is associated with the optimization of physicochemical and pharmacokinetic properties of the lead compounds with their balanced multitarget profile unchanged. In some cases it might be possible to design a multitarget profile into a single chemical entity by linking two agents, or by simultaneously optimizing dual specificity into a single low-molecular-weight compound. Other challenges and difficulties in designing successful multitarget drugs would include the limited knowledge on the target pairs

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**Figure 1.** Chemical structures of LTA4, LTB4, and LTC4. The substrate and products of LTA4H and LTC4S enzymes.

that can interact synergistically, selection of targets for which the drugs are designed, achievement of multiselective action without being nonselective, and the management of counterbalancing effect of biological pathways to resist the actions upon drug administration.<sup>3,4</sup> With an understanding of the importance of multitarget drugs, around 20 drugs were approved or in advanced development stages.<sup>10</sup> A very few computer-aided multitarget methodologies such as sequential docking and combining molecular docking with common pharmacophore mapping were employed in designing multitarget drugs. Sequential docking methodology is computationally expensive for large-scale database screening<sup>11</sup> whereas a study explaining the methodology combining molecular docking with pharmacophore mapping used only a single conformation inhibitor–protein complex.<sup>2</sup> Thus, a predefined strategy merging the information of necessary chemical features from experimentally known inhibitors as well as from the available interaction points at the binding sites of multiple targets to identify the small molecules modulating multiple targets is much needed. In view of this, a new strategy combining ligand-based pharmacophore models of multiple targets and the receptor-based information to discover multitarget inhibitors is proposed in this study.

The development and maintenance of inflammatory response are regulated by a complex network of cellular and soluble factors.<sup>12</sup> Production of the biologically active metabolites leukotrienes (LT), which are involved in various inflammatory diseases, is initiated by the conversion of arachidonic acid (AA) through the 5-lipoxygenase pathway.<sup>13</sup> LTA4, an important epoxide intermediate, is formed and converted either by stereoselective hydration of LTA4 hydrolase (LTA4H) to LTB4 or glutathione addition by LTC4 synthase (LTC4S) to LTC4 (Figure 1).<sup>14</sup> Further enzyme actions convert LTC4 to LTD4 and then to LTE4 which are known to mediate the inflammation and immediate hypersensitivity reactions.<sup>15</sup> LTB4 can cause tissue damage and other diseases by activating inflammatory cells.<sup>16</sup> Elevated levels of LTB4 have been involved in inflammatory diseases including psoriasis. LTB4 is a very potent chemotactic agent, inducing neutrophil adherence to endothelial cells, degranulation, and modulation of cytokinin production. LTA4H catalyzes the final rate limiting step in the biosynthesis of LTB4, a potent lipid chemoattractant involved in inflammation, immune responses, host defense against infection, platelet activating factor induced shock, and lipid homeostasis.<sup>12,17</sup> LTA4H is a bifunctional zinc-dependent metalloenzyme that integrates the specific epoxide hydrolase activity for the fatty acid substrate LTA4 and aminopeptidase activity for which the biological role has not been validated yet. Thus, inhibition of LTA4H to decrease the production of LTB4 may have

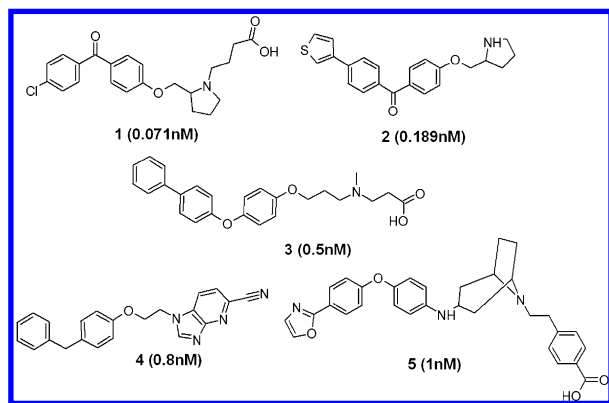
therapeutic potential in all the diseases caused by LTB4.<sup>16</sup> LTC4S is a specialized membrane-bound glutathione transferase enzyme that conjugates the glutathione (GSH) moiety to LTA4 and converts it to LTC4, one of the cysteinyl leukotrienes (LTC4, LTD4 and LTE4), but not the xenobiotics like other microsomal glutathione transferases (MGST2 and MGST3).<sup>18</sup> A variety of small molecule inhibitors are reported for the inhibition of hLTA4H, and only a few are available for hLTC4S. Both of these enzymes, by different mechanisms, process the same substrate LTA4 and produce different products that are involved in inflammatory responses. Inhibition of either one of these two enzymes would allow the other product to be derived and available to mediate the inflammatory response. Thus, the discovery of a new small molecule inhibitor that can inhibit both enzymes processing LTA4 would be beneficial in treating various inflammatory diseases.

In view of this, a new strategy that combines the advantage of pharmacophore modeling and molecular docking has been employed to identify the small molecules containing the important chemical features to inhibit two enzymes hLTA4H and hLTC4S, which are involved in LTA4 processing to yield various endogenous leukotrienes to mediate the inflammatory responses. This strategy has been successfully applied to identify the compounds from the chemical database that can strongly bind at the active site of both the targets and thereby act as competitive inhibitors to the substrate, LTA4. Finally, eight druglike compounds from the database are reported as possible dual-inhibitors for hLTA4H and hLTC4S enzymes.

## METHODS AND MATERIALS

**Construction of Druglike Database.** Maybridge, a commercial chemical database containing 59 652 compounds, has been used in this study.<sup>19</sup> However, this database is found to have number of nondruglike compounds. As it is meaningless to dock all the compounds of this database into the active site of protein target and then reject them in the later stage for their nondruglike properties, the compounds not satisfying druglike properties were excluded from the database prior to molecular docking (structure-based virtual screening). In order to achieve this step, compounds in the Maybridge database were subjected to various rigorous druglike filters such as Lipinski's rule of five and ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties. *Prepare Ligands* and *ADMET Descriptors* protocol as available in Accelrys Discovery Studio 2.5 (DS) program were used in this step.

**Target Structure Preparation.** In this study, we have used the crystal structure of hLTA4H (PDB code 3FH7) bound with an inhibitory molecule (4-[(2S)-2-[[4-(4-chlorophenoxy)phenoxy]methyl]pyrrolidin-1-yl]butanoate) and the crystal structure of hLTC4S (PDB code 2PNO) with a substratelike molecule (dodecyl- $\beta$ -D-maltoside, DDM).<sup>15,20,21</sup> Both the enzymes act on the same substrate, LTA4, and result in two closely related chemical compounds LTB4 and LTC4, which are involved in inflammatory response (Figure 1). hLTC4S is a homotrimer where the LTA4 binding site is located between two monomers.<sup>15</sup> The 3D coordinates of these enzymes were obtained from PDB and processed further by removing all the water molecules as well as other

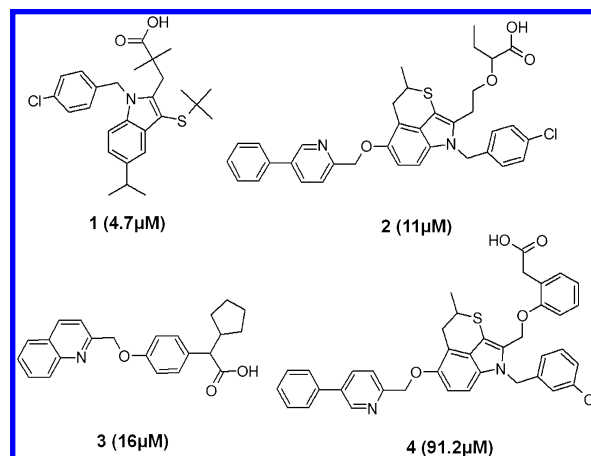


**Figure 2.** Training set compounds used in hLTA4H pharmacophore modeling. The experimental activity values (IC<sub>50</sub>) are given in parentheses.

heteroatoms except the metal atom and the bound inhibitor in hLTA4H whereas in hLTC4S all the heteroatoms were removed except the glutathione moiety. Hydrogen atoms were added to the target protein structures using CHRAMM<sup>22</sup> force field as available in DS followed by the minimization of added hydrogen atoms using *Smart Minimizer* protocol with a constraint on heavy atoms. The resulting target protein structures were used in molecular docking studies.

**Molecular Docking for LTA4H Inhibition.** Virtual screening is a rapid process to identify a subset of compounds, which can be the possible structural leads for drug designing, from a huge collection. In this study, a locally constructed druglike Maybridge database containing a huge set of diverse compounds was used for docking into the active site of the hLTA4H enzyme. Molecular docking program GOLD (Genetic Optimization for Ligand Docking) 4.1 from Cambridge Crystallographic Data Centre, U.K., was used to dock the database of compounds into a defined active site.<sup>23</sup> GOLD uses a genetic algorithm to dock the small molecules into the protein active site. It allows the full range of flexibility of ligands and partial flexibility of protein. Protein coordinates from the crystal structure complex of hLTA4H with a bound inhibitor determined at a resolution of 2.05 Å were utilized to define the active site. The active site was defined with 10 Å radius around the bound inhibitor. Top 10 scored conformation of every ligand was allowed to be saved at the end of the calculation. The early termination option was used to skip the genetic optimization calculation when any five conformations of a particular compound predicted within the rmsd value of 1.5 Å. GOLD fitness score is calculated from the contributions of hydrogen bond and van der Waals interactions between protein and ligand, intramolecular hydrogen bond, and strain of the ligand.<sup>23,24</sup> All other parameters were kept at their default values.

**Common Feature Pharmacophore Generation for hLTA4H.** Chemical compounds with the experimental hLTA4H inhibition were obtained from various scientific resources, and a small database was generated. Five diverse compounds with the IC<sub>50</sub> values less than or equal to 1 nM were chosen as training set and employed in common feature pharmacophore generation calculation (Figure 2).<sup>21,25–27</sup> The binding conformations of all the five inhibitors are known X-ray crystallographically, and their coordinates are available in the PDB. It is assumed that the chemical compounds used in developing ligand-based pharmacophore models using either common feature or structure–activity relationship



**Figure 3.** Training set compounds used in hLTC4S pharmacophore modeling. The experimental activity (IC<sub>50</sub>) values are given in parentheses.

approach bind the same binding site of the target protein. All the calculations were performed in DS. A principal value of 2 and maximum omit feature value of 0 were assigned to the most active compound in the training set whereas 1 and 0 were assigned for the other compounds to label them as moderately active. Diverse conformations were generated for the training set compounds using *Diverse Conformation Generation* protocol with the parameters of 250 conformations with the energy cut off range of 20 kcal/mol above the global minimum. Minimum interfeature distance was set to 2 Å from the default value of 2.97 Å in order to call the *Common Feature Pharmacophore Generation* protocol to consider the functional groups that are present in the training set compounds as closely as 2 Å. This has been done mainly to include the closely related functional groups (C=O and OH) in carboxylic acid which is present in most of the training set compounds to be involved in metal coordination. *Feature Mapping* protocol was used to identify the common chemical groups present in the training set compounds. As predicted, hydrogen bond acceptor (HBA), hydrogen bond donor (HBD), hydrophobic (HY), positive ionizable (PI), negative ionizable (NI), and ring aromatic (RA) features were selected during the pharmacophore generation.

**Common Feature Pharmacophore Generation for hLTC4S.** Several scientific resources were searched for the compounds with the experimentally known inhibitory values for hLTC4S for which not many compounds were tested experimentally. Four experimentally tested compounds with considerable structural diversity were chosen to be the training set to build a common feature pharmacophore model for hLTC4S (Figure 3).<sup>28,29</sup> All the calculations were done in DS with the parameters used for hLTA4H. *Feature Mapping* protocol predicted HY, HBA, RA, and NI functionalities as the most found features in the training set and those used in pharmacophore generation. The most active compound in the training set was given the principal value of 2 and maximum omit feature value of 0 whereas the other compounds were assigned with 1 and 0, respectively, on the basis of the activity values.

**Ligand Pharmacophore Mapping.** The hit compounds obtained from the molecular docking experiment performed for hLTA4H inhibition with the druglike Maybridge database are used in this experiment along with the common feature pharmacophore models generated for hLTA4H and hLTC4S.



**Ligand Pharmacophore Mapping** protocol as implemented in DS is used to map all the hit compounds upon both the pharmacophore models with the *Best Flexible Search* option. The compounds that mapped well on both the pharmacophore models were chosen as possible hits for dual inhibition and utilized in molecular docking of hLTC4S enzyme.

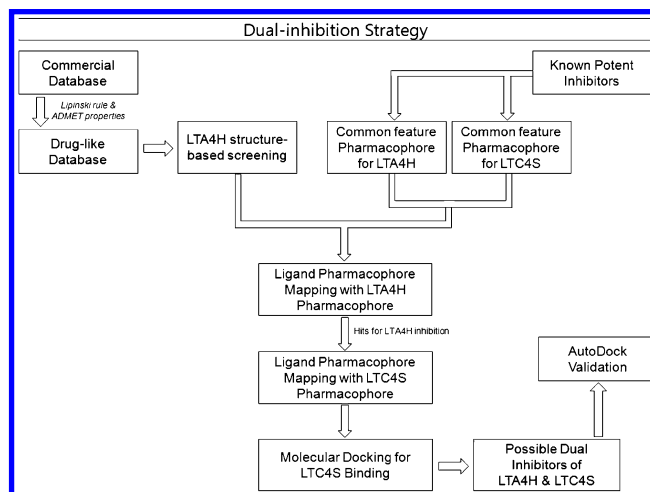
**Molecular Docking of Possible Dual-Inhibition Leads into LTC4S.** The coordinates of the prepared crystal structure of hLTC4S which is devoid of the water molecules and other heteroatoms except the bound glutathione have been used as the target in this molecular docking experiment. The active site was defined around the thiol (SH) group of glutathione with the radius of 10 Å. The compounds that mapped well on both the pharmacophore models were used as ligands. All other parameters were kept at the same values as used in molecular docking for the hLTA4H enzyme.

**Molecular Docking Validation Using Autodock 4.2.** We used Autodock 4.2 to check the binding energies of the chosen compounds at the active sites of hLTA4H and hLTC4S. Grid maps of different grid points, centered on the ligands of the complex structures, were used for hLTA4H and hLTC4S, respectively, to cover the binding pockets. A spacing of 0.375 Å was set, and a Lamarckian genetic algorithm was used for all molecular docking simulations. Population size of 150, mutation rate of 0.02, and crossover rate of 0.8 were set as the parameters. Simulations were performed using up to 2.5 million energy evaluations with a maximum of 27 000 generations. Each simulation was performed 10 times, yielding 10 docked conformations.<sup>30</sup> The lowest energy conformations were regarded as the binding conformations between the ligands and the proteins.

**Reverse Validation.** In this validation process, the complete strategy followed in this study was reversed to ensure that the identified hits really fit the generated pharmacophore models and active sites of both targets. All the parameters required for molecular docking and pharmacophore mapping were set as used in actual process.

## RESULTS AND DISCUSSION

**Dual-Inhibitor Design Strategy.** The pharmacophore modeling procedure, which has been a great success in medicinal chemistry, was utilized in this study.<sup>31,32</sup> There are two different pharmacophore modeling approaches widely used in computer-aided drug discovery research. The first approach is based on a set of ligands with experimentally known inhibition for a particular therapeutic target whereas the second one exploits the receptor-based information for designing pharmacophore models.<sup>33–35</sup> Our strategy is based on the information extracted from the ligand- and receptor-based approaches to identify the dual-inhibitors. Most of the compounds identified to bind well at the active site of a particular protein by virtual screening procedure are predicted to be false positives when screened experimentally. Some researchers plead that this fault is due to the scoring functions that are used to rank the predicted poses while others say that the docking program does not always produce correct poses. This problem can be addressed by postdocking pharmacophore filtering which can eliminate the compounds from virtual screening if they are chemically incompatible with the active site. This strategy, used in the current study, can be a valid one as it merges the strength of ligand-based



**Figure 4.** Flowchart explaining the strategy used in this study.

(pharmacophore modeling) and receptor-based (molecular docking) approaches together (Figure 4).

As a first step, a druglike database has been prepared and docked into the active site of one of the two target proteins to predict the binding affinity and molecular interactions of small molecules in the database. The compounds that formed strong interactions at the active site were chosen for the next step. The second step involves the pharmacophore generation for both the target proteins using their experimentally known inhibitors. In the third step, the docking hits of the first target protein were mapped over the derived pharmacophore models to filter the compounds scoring the best fit values and also to check whether the binding conformations of compounds accommodate the derived pharmacophore models. The compounds that fit well upon both the pharmacophore models were docked into the active site of the second protein target to observe their binding. The compounds that bind strongly at both the active sites were considered as hits to design dual-inhibitors.

**Molecular Docking of Druglike Database for hLTA4H Binding.** Maybridge, a chemical database containing 59 652 diverse chemical compounds, was screened using various druglike parameters in order to remove the nondruglike compounds. *Prepare Ligands*, *Molecular Properties* and *ADMET Descriptors* options available in DS were used for this purpose. *Prepare Ligands* has removed all the duplicate structures as well as the compounds violating Lipinski's rule of five and also generated 3D conformations of all the compounds. After the rejection of 11 155 compounds violating any one of the Lipinski's rules, with bad valency and duplicate structures, 48 497 compounds were selected for the next level of screening. *ADMET Descriptors* protocol has calculated various parameters related to the bioavailability and toxicity of compounds such as absorption level and hepatotoxicity, etc., for all 48 497 compounds. The compounds with good absorption level (level 0 according to DS), good and optimal solubility (level 3 and 4), low blood brain barrier penetrability (level 3), CYP450 2D6 noninhibition, and nonhepatotoxic nature were selected as druglike compounds. Finally, 4966 druglike compounds were selected and used subsequently in molecular docking study. The preprocessed structure of hLTA4H for molecular docking study, as discussed in target structure preparation process, was used as protein target to dock all the druglike compounds by the

**Table 1.** Summary of the Pharmacophore Models Generated for LTA4H

hypothesis	features <sup>a</sup>	rank	direct hit <sup>b</sup>	partial hit <sup>c</sup>	max. fit
Hypo1	RRHAA	64.048	11111	00000	5
Hypo2	RRHAA	64.048	11111	00000	5
Hypo3	RRHAA	64.048	11111	00000	5
Hypo4	RRHAA	64.048	11111	00000	5
Hypo5	RRHAA	64.037	11111	00000	5
Hypo6	RRHAA	63.747	11111	00000	5
Hypo7	RRHAA	63.434	11111	00000	5
Hypo8	RRHAA	63.434	11111	00000	5
Hypo9	RRHAA	63.226	11111	00000	5
Hypo10	RRHAA	63.226	11111	00000	5

<sup>a</sup> R, ring aromatic; H, hydrophobic; A, hydrogen bond acceptor.

<sup>b</sup> Direct hit represents whether (1) or not (0) a molecule is mapped on the pharmacophore model. <sup>c</sup> Partial hit represents whether (1) or not (0) a molecule is mapped all but one features in the pharmacophore model.

GOLD program. All the compounds were successfully docked without any error during *genetic optimization* run. The compounds in the training set of hLTA4H were also docked with the same parameters used in database docking. Compound 1 has scored a GOLD fitness score of 81.332 along with the strong molecular interactions with the active site components. The 300 hit compounds from the druglike database scoring a GOLD fitness score greater than 70 were retained as possible hLTA4H inhibitors and utilized in pharmacophore filtering.

#### Generation and Selection of Pharmacophore Models.

Common feature pharmacophore models were generated for both the target proteins using sets of experimentally known inhibitors. *Common Feature Pharmacophore Generation* protocol implemented in DS has been utilized in this study.

**Pharmacophore Model for hLTA4H.** Five compounds with the experimentally tested IC<sub>50</sub> values less than or equal to 1 nM for LTA4H inhibition were selected as a training set to generate a valid common feature pharmacophore model. The training set includes diverse compounds in terms of their chemical structures. Most of these compounds possess a carboxylic group or other hydrogen bond acceptor moieties as metal binding group. The most active compound in the training set possesses the potent activity value of 0.5 nM. The compounds with their associated conformational models were submitted to DS. Several common feature pharmacophore generation runs were carried out by varying the control parameters such as principal, maximum omit feature, minimum interfeature distance values, and the combination of pharmacophoric features in order to obtain a best model. The features selected for this pharmacophore generation were HBA, HBD, HY, PI, and NI. Ten pharmacophore models (Hypo 1–10) were generated by DS, and the results were analyzed (Table 1). The ranking scores for these 10 pharmacophore models ranged from 64.048 to 63.226. All of the generated pharmacophore models are composed of five features that include two RA, one HY, and two HBA features. The top four pharmacophore models scored the same ranking scores and same molecular features. Analysis of best fit values of the training set compounds toward these models was performed to choose the best model (Table 2). All of these four pharmacophore models were found to have the same features with the same interfeature distances but differed only in the orientation of the vectors present in HBA

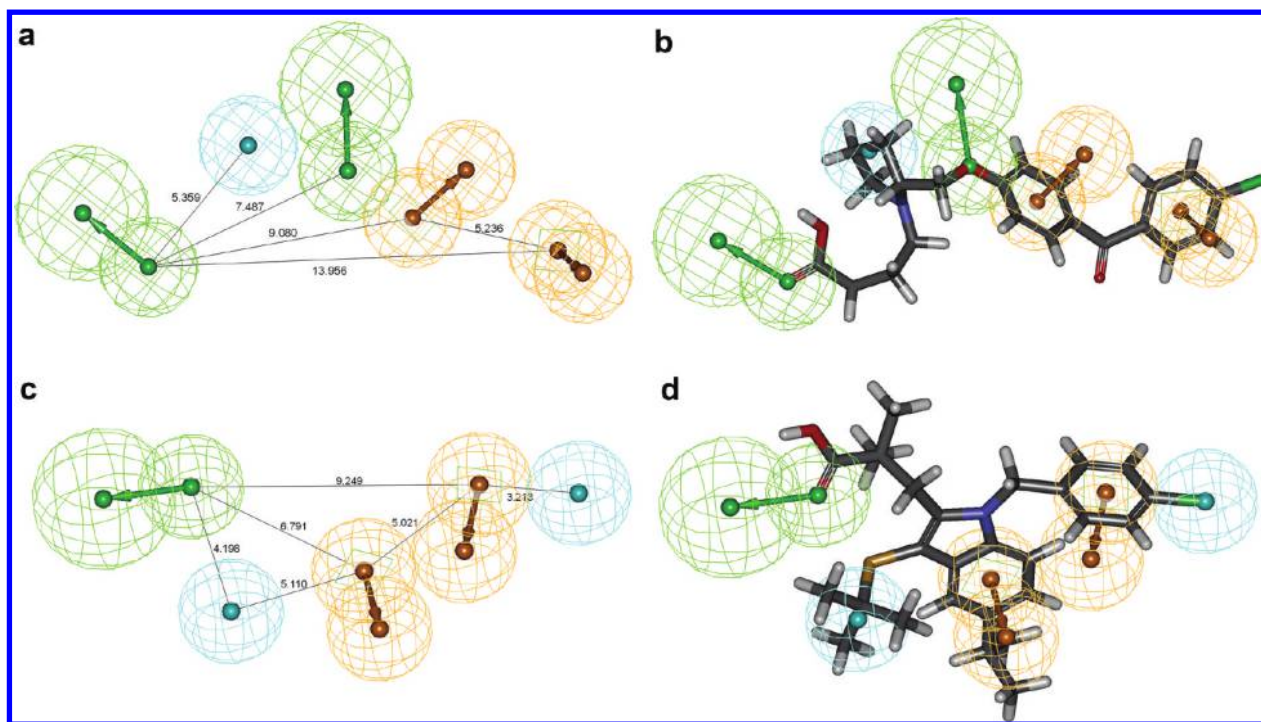
**Table 2.** Best Fit Values of the Training Set Compounds Based on First Five Pharmacophore Models Generated for LTA4H

compound	fit value				
	Hypo 1	Hypo 2	Hypo 3	Hypo 4	Hypo 5
1	4.999	4.999	4.999	4.999	4.999
2	3.140	1.578	0.721	0.996	1.344
3	2.469	2.846	2.189	2.033	4.078
4	1.512	1.553	1.666	2.401	2.027
5	1.479	2.384	1.103	1.260	2.719

and RA features. This analysis revealed that the same pharmacophore model has been repeatedly generated from the training set compounds. Thus, on the basis of the best fit values and the ranking score, Hypo 1 (LH-Hypo1) has been selected as the best model for further studies (Figure 5a). The overlay of compound 1 on pharmacophore model LH-Hypo1 (Figure 5b) showed that two phenyl groups mapped over two RA and terminal carboxyl moiety and the oxygen atom connecting the phenyl and pyrrolidine moieties mapped two HBA features whereas the pyrrolidine mapped over the only HY feature.

**Pharmacophore Model for hLTC4S.** The pharmacophore model for hLTC4S was built using a set of 4 compounds with diverse chemical structures and DS program. All the compounds in the training set contain a carboxylic moiety to bind specifically with a particular amino acid residue in the active site of hLTC4S. The experimental hLTC4S inhibitory values of training set compounds are less than 100  $\mu$ M. The compounds with their diverse conformational models were used in the pharmacophore generation process using DS. Several pharmacophore models were computed with different control parameters in order to generate a valid one. The most active compound in the training set was given a principal value of 2 and maximum omit feature value of 0. HY, HBA, RA, and NI features were used in pharmacophore generation. Common feature pharmacophore generation run resulted in 10 pharmacophore models (Table 3). The ranking scores of these 10 pharmacophore models ranged from 53.492 to 52.267. All the 10 pharmacophore models comprised five pharmacophoric features, and eight of them comprised two RA, one HBA, and two HY features, whereas the remaining two models were made of an additional HBA feature in place of an RA feature. As the top four generated pharmacophore models have scored a ranking score relatively close to each other, an analysis of the best fit values of the training set compounds scored against the generated pharmacophore models to choose the best model was carried out (Table 4). The calculated best fit values indicated Hypo 1 (LS-Hypo1) as the best model and thus was chosen to be used in further study (Figure 5c). Compound 1 in the training set of hLTC4S overlaid well on LS-Hypo1 (Figure 5d).

**Validation of Pharmacophore Models.** The reliability of the generated pharmacophore models was validated on the basis of the presence of the chemical features necessary to interact with the key amino acid residues in the active site of the corresponding target protein. The pharmacophore model generated for hLTA4H inhibitors has been evaluated to possess the required features that are complementary to the active site. This pharmacophore was made of two RA and two HBA features along with an HY feature. Ligand interaction diagram generated for the hLTA4H–inhibitor



**Figure 5.** Generated pharmacophore models and overlays: (a) pharmacophore model for hLTA4H along with its interfeature distance constraints, (b) overlay of the most active compound in the training set of hLTA4H, (c) pharmacophore model for hLTC4S along with its interfeature distance constraints, and (d) overlay of the most active compound in the training set of hLTC4S.

**Table 3.** Summary of the Pharmacophore Models Generated for LTC4S

hypothesis	features <sup>a</sup>	rank	direct hit <sup>b</sup>	partial hit <sup>c</sup>	max. fit
Hypo1	RRAHH	53.492	1111	0000	5
Hypo2	RRAHH	53.137	1111	0000	5
Hypo3	RRAHH	53.137	1111	0000	5
Hypo4	RRAHH	53.088	1111	0000	5
Hypo5	RRAHH	52.791	1111	0000	5
Hypo6	RRAHH	52.649	1111	0000	5
Hypo7	RAHHA	52.369	1111	0000	5
Hypo8	RAHHA	52.369	1111	0000	5
Hypo9	RRAHH	52.267	1111	0000	5
Hypo10	RRAHH	52.267	1111	0000	5

<sup>a</sup> R, ring aromatic; A, hydrogen bond acceptor; H, hydrophobic.

<sup>b</sup> Direct hit represents whether (1) or not (0) a molecule is mapped on the pharmacophore model. <sup>c</sup> Partial hit represents whether (1) or not (0) a molecule is mapped all but one features in the pharmacophore model.

**Table 4.** Best Fit Values of the Training Set Compounds Based on First Four Pharmacophore Models Generated for LTC4S

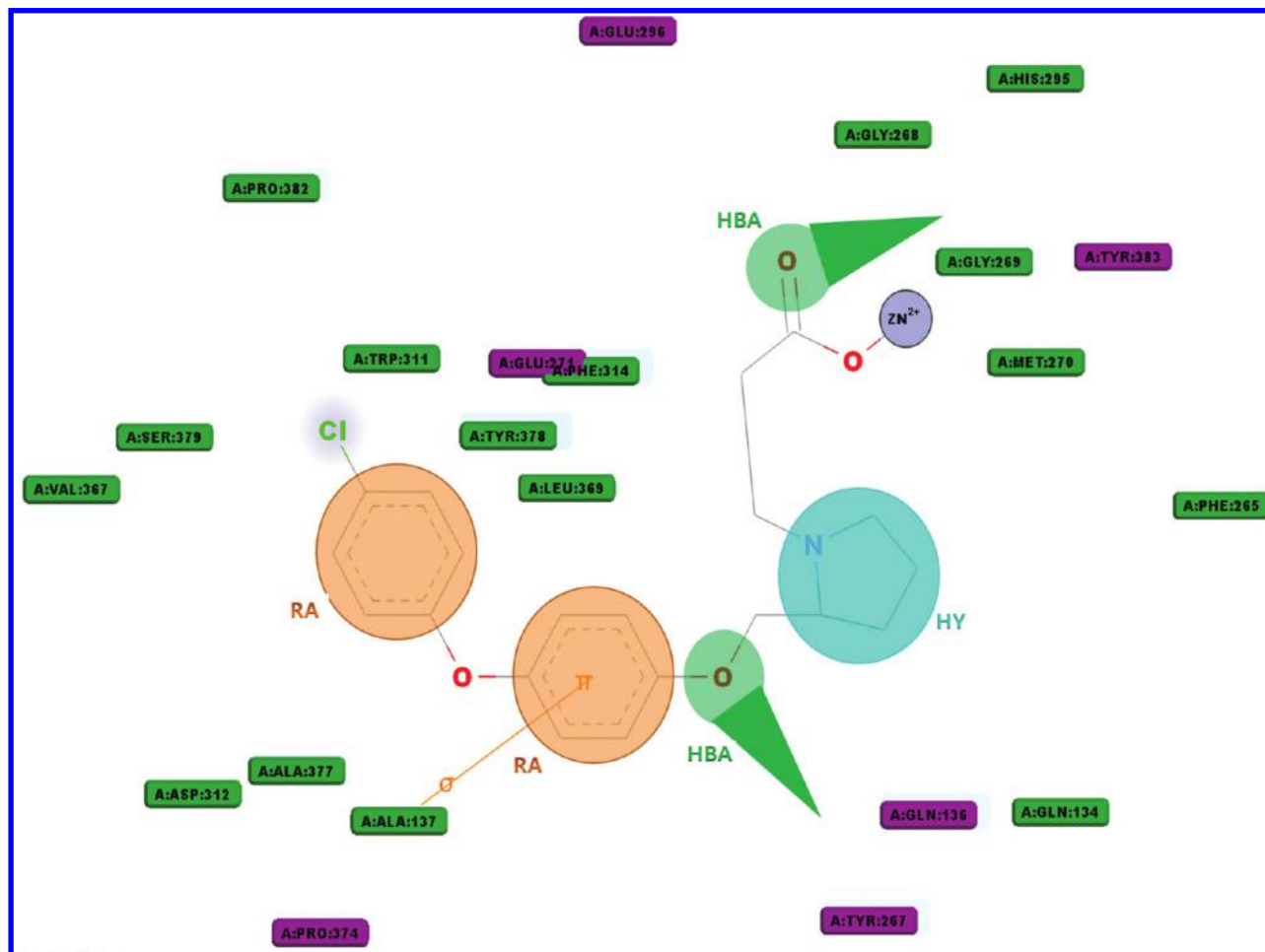
compound	fit value			
	Hypo 1	Hypo 2	Hypo 3	Hypo 4
1	4.999	4.999	4.999	4.999
2	4.220	3.575	4.223	3.884
3	4.063	2.850	3.266	3.609
4	1.613	1.288	0.765	1.823

complex (PDB code 3FH7) using DS has revealed the amino acids that are complemented by every feature present in the pharmacophore model (Figure 6). Overlay of the bound inhibitor on the LH-Hypo1 pharmacophore model revealed that the RA features were found to interact hydrophobically with A137, W311, V367, L369, A377, and Y378 whereas the HY feature present in a position to interact with F265, M270, H295, and other residues. HBA features present in

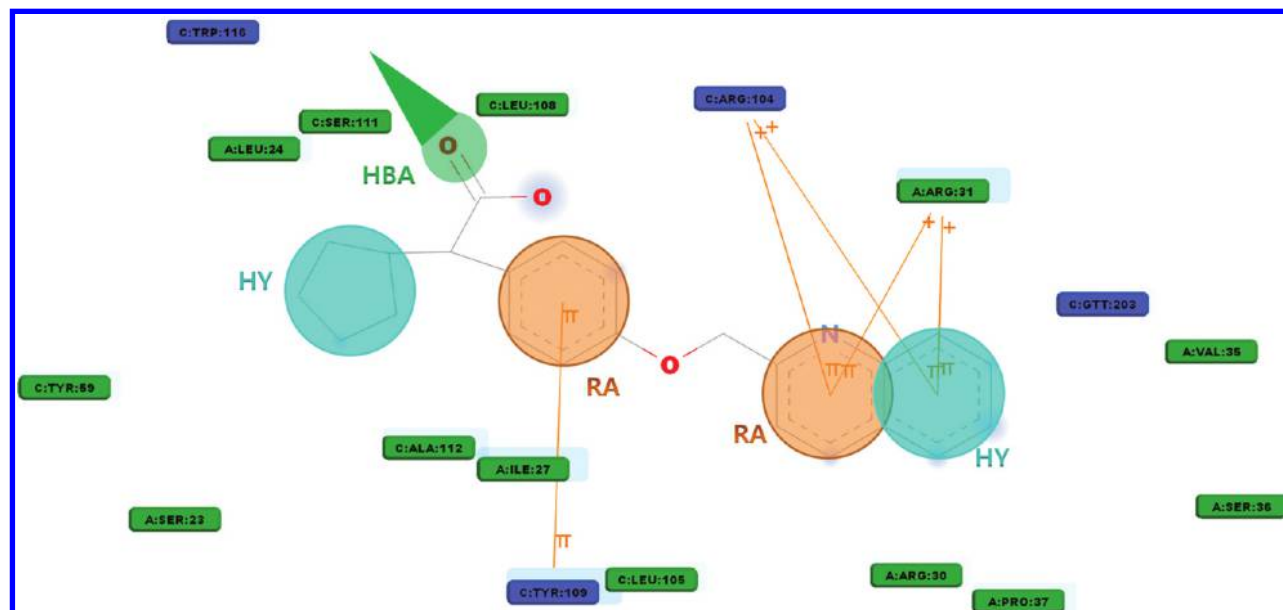
the pharmacophore model were located in such a way to interact with the metal ion and other active site residues such as H295, E296, Y383, and others. In terms of the pharmacophore model developed for hLTC4S inhibitors, the overlay of one of the training set compounds and its binding conformation from the molecular docking results revealed that the two RA and HY features have formed a number of hydrophobic contacts to amino acids such as L24, I27, Y59, L108, Y109 and other residues from chain A and R30, R31, V36 and other residues from chain C. The only negative ionizable carboxyl group present in the compound is found to be present close to a positive ionizable residue R104 which is one of the important amino acids for positioning the epoxy moiety of the biological substrate, LTA4 (Figure 7). These observations related to the features present in the developed pharmacophore models compared with the interaction points at the active site have shown the significance of the developed pharmacophore models.

**Pharmacophore-Based Filtering of Database Hits.** The binding conformations of 300 compounds that scored a GOLD fitness score greater than 70 were initially mapped over the pharmacophore model, LH-Hypo1, generated for hLTA4H. Maximum omitted feature value was set to 0 during the pharmacophore mapping process. Forty compounds mapped all the pharmacophoric features present in LH-Hypo1, and 28 compounds scoring a fit value greater than 2.5 were saved to use further. The fit value restriction was fixed based on the fit values of the training set compounds. These compounds were then mapped over the pharmacophore model LS-Hypo1 that was generated for the second target LTC4S. The maximum omitted feature value was set to 1 in order to increase the probability of retrieving more compounds for molecular docking. In the end, 27 of 28 compounds could map the LS-Hypo1 effectively. These 27 compounds along with four hLTC4S training set com-





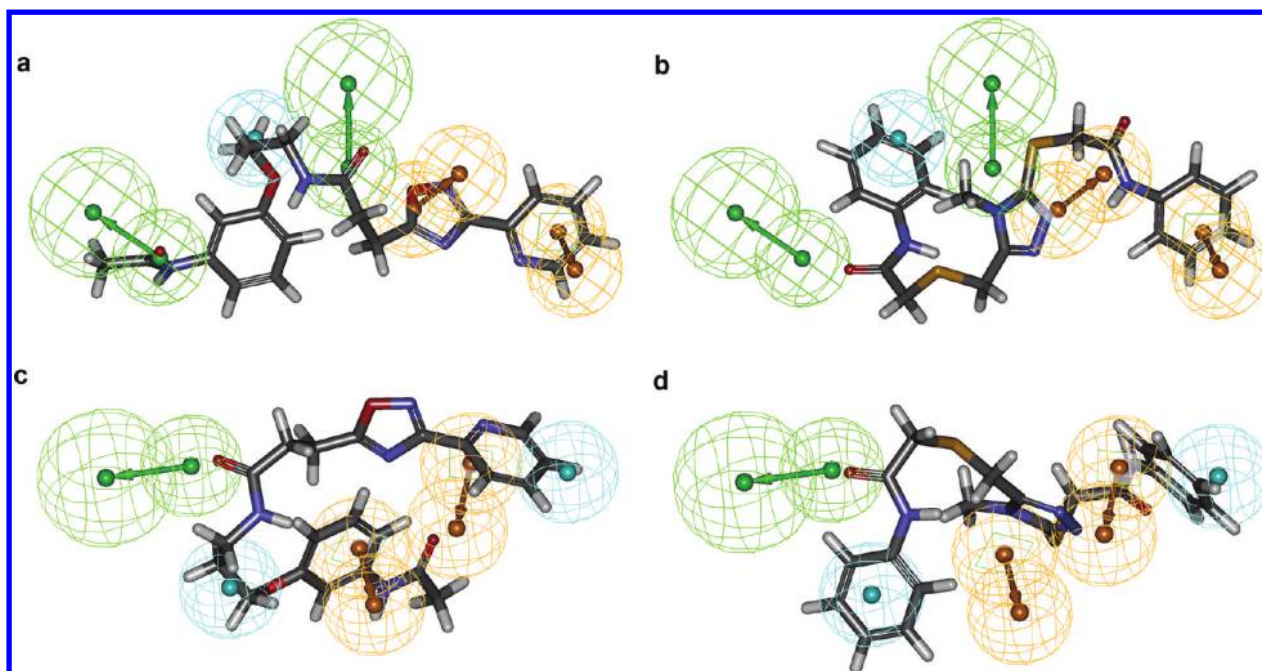
**Figure 6.** Ligand–protein interaction diagram from the hLTA4H–inhibitor (compound 1 in the training set of hLTA4H) complex (PDB code 3FH7). The pharmacophore mapping of the same compound is also illustrated. HBA, hydrogen bond acceptor; HY, hydrophobic; RA, ring aromatic. The locations of amino acid residues are represented in rectangular boxes, where purple and green colors denote both the hydrogen bond acceptor/donor and nonpolar contacts, respectively.



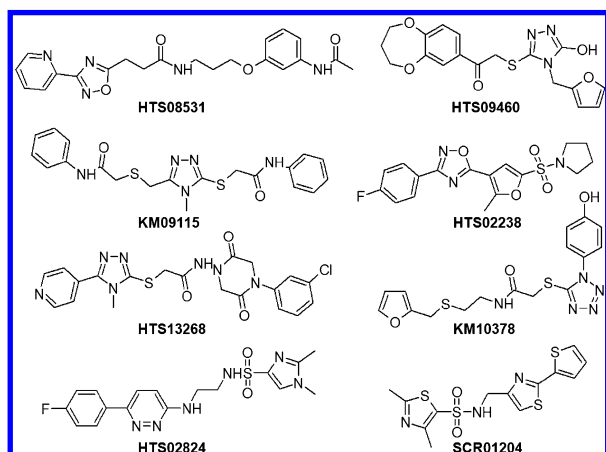
**Figure 7.** Ligand–protein interaction diagram from the hLTC4S–inhibitor (best docked conformation of the most active compound in the training set of hLTC4S) complex. The pharmacophore mapping of the same compound is also illustrated. HBA, hydrogen bond acceptor; HY, hydrophobic; RA, ring aromatic. The locations of amino acid residues are represented in rectangular boxes, where purple and green colors denote both the hydrogen bond acceptor/donor and nonpolar contacts, respectively.

pounds were docked into the active site of hLTC4S to predict their binding modes and protein–ligand interactions. Figure

8 represents the pharmacophore overlay of two hit compounds upon both the pharmacophore models.



**Figure 8.** HTS08531 and KM09115 of the 8 identified leads for dual-inhibition are overlaid on LH-Hypo1 (a and b) and LS-Hypo1 (c and d) pharmacophore models, respectively.



**Figure 9.** Chemical structures of identified hits for dual inhibition.

**Molecular Docking for hLTC4S Binding.** The crystal structure of hLTC4S was prepared for molecular docking as explained in the target structure preparation process. The compounds from the pharmacophore filtering along with the training set compounds were docked into the defined active site around the thiol group of the glutathione moiety using the GOLD program. The most active compound 1 (MK886) in the training set has scored the GOLD fitness score of 44.93, and interestingly all the 27 hit compounds have scored better than MK886 at the binding site. The binding modes of all the docked compounds were analyzed for their molecular interactions at the active site.

On the basis of the binding mode analysis and structural diversity, eight compounds that bind both the protein targets with good binding characteristics were listed as possible dual inhibitors (Figure 9) for these enzyme targets that are involved in the inflammatory process.

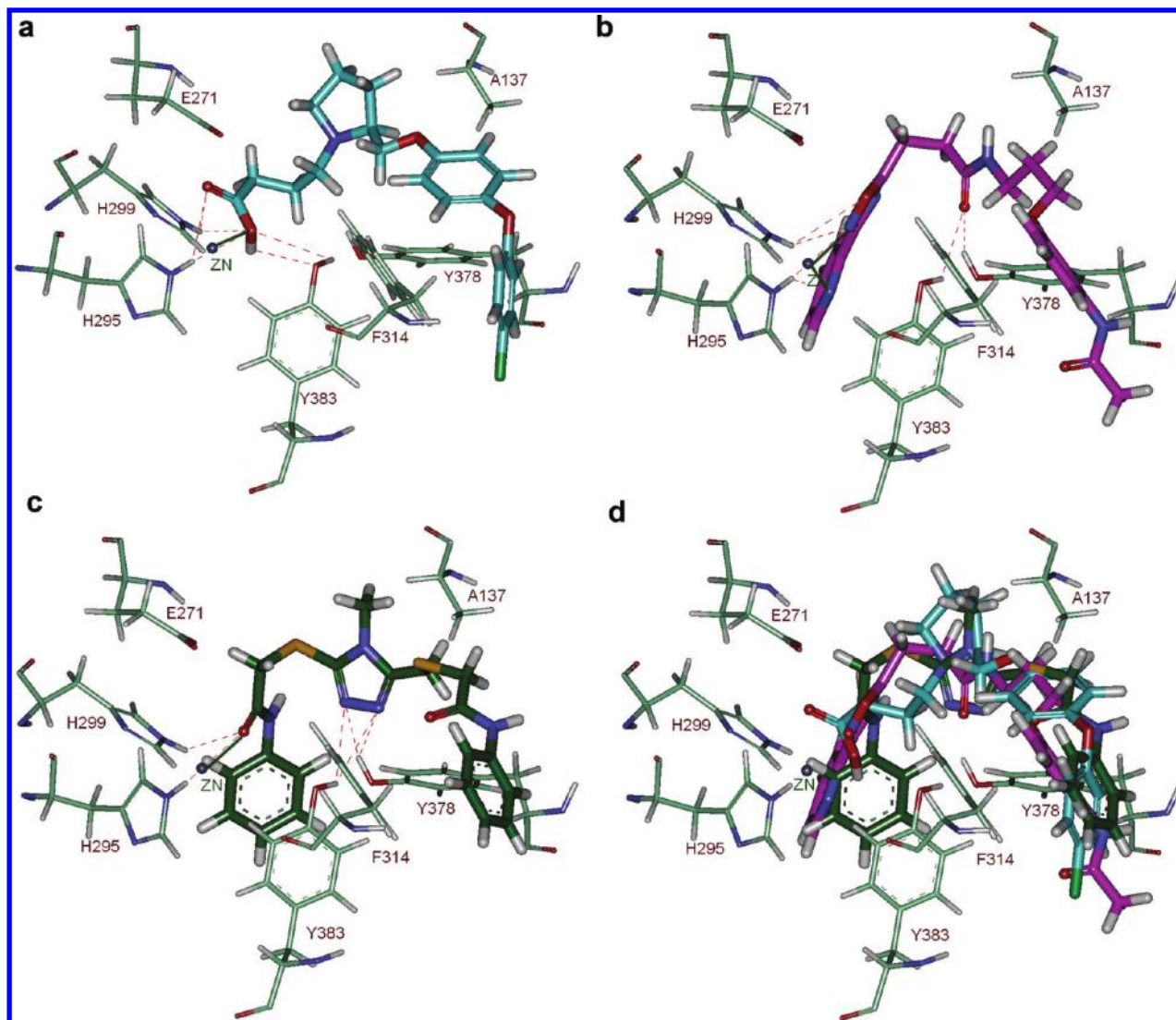
**Validation of Molecular Docking Using Autodock 4.2.** The binding modes of the proposed possible dual inhibitors for hLTA4H and hLTC4S were further analyzed using Autodock 4.2. This program takes more time but

predicts the binding conformations more accurately and results in the binding energy of each docked compound. In terms of hLTA4H docking using Autodock, all the 8 compounds have scored similar or better binding energy values compared to the most active compounds in the training set. Autodock docking of the final eight compounds for hLTC4S inhibition also produced better results when compared to the most active compounds in the training set.

**Binding Modes of the Identified Hits.** A good set of crystal structure complexes bound with a different set of inhibitors is available for hLTA4H. The molecular interactions between the target protein (hLTA4H) and the different inhibitors were observed from the ligplot figures available via the PDBSum database.<sup>36,37</sup> Compound 1 in the training set of hLTA4H has shown hydrogen bond interactions with H295, H299, and Y383.<sup>21</sup> It has also formed a strong covalent bond with the metal ion ( $\text{Zn}^{2+}$ ) in the active site of hLTA4H. This compound has scored a GOLD fitness score of 81.332. The binding mode and the molecular interactions of this compound were considered as reference to assess the binding modes of the hit compounds (Figure 10a).

There is no crystal structure solved so far for hLTC4S with a bound inhibitory molecule. In order to predict the binding mode of a known inhibitor, MK886 with an  $\text{IC}_{50}$  value of  $0.4 \mu\text{M}$  has been docked using the GOLD program. The binding modes and the molecular interactions of the hit compounds were compared with the result of MK886 (Figure 11a). This compound has scored a GOLD fitness score of 44.935 and formed hydrogen bond interactions with Y59 and Y109 residues. Ideally positioned R104 is considered as a very important amino acid to promote the  $\text{pK}_a$  shift of the thiol group of GSH through its positive charge and thereby plays a key role in activating the thiol group for a nucleophilic attack on the LTA4 epoxide. It is also located close to the probable position of the C5 atom of the substrate LTA4 and thereby assists the stabilization of the substrate anion. In addition, another amino acid residue R31 located on the





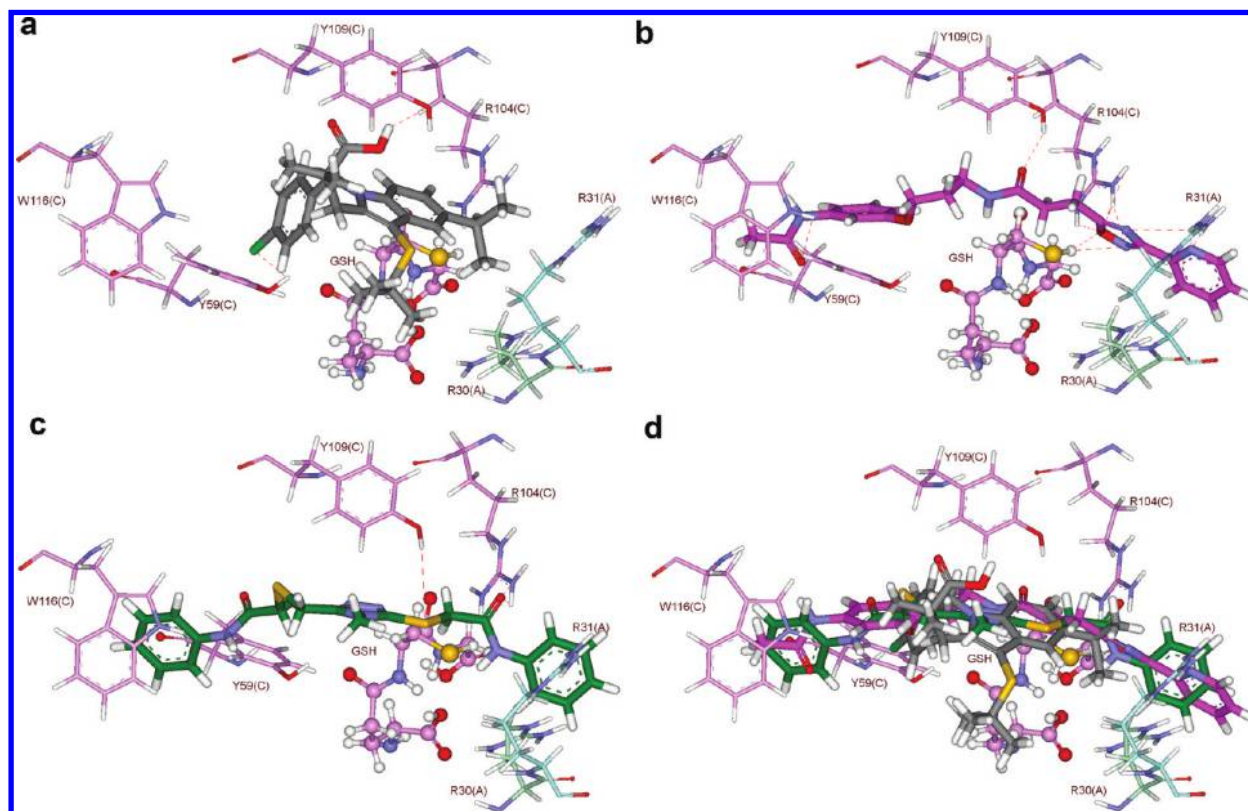
**Figure 10.** Binding poses of (a) compound 1 from the training set of hLTA4H, (b) HTS08531, and (c) KM09115, and (d) overlay of all three compounds in the active site of hLTA4H. Protein residues and ligands are represented by thin and thick sticks, respectively. The metal ion (Zn) is shown in gray color and ball in shape. Hydrogen bonds and coordination bonds are shown in red dashed and green solid lines, respectively.

distal side of the substrate may also assist in substrate anion stabilization.<sup>15</sup> These amino acid residues along with the GSH moiety were considered very important for the main function of the target protein to act upon LTA4, the substrate.

The 8 hit compounds that were chosen from the molecular docking and pharmacophore filtering studies are HTS08531, HTS09460, KM09155, HTS02238, HTS13268, KM10378, HTS0824, and SCR01204 (Figure 9). These final compounds were chosen on the basis of the observed molecular interactions with the respective important residues from hLTA4H and hLTC4S (Figures 10 and 11). The GOLD fitness score and the calculated binding energy values for all the final hits are given in Table 5. The binding modes and molecular interactions between some of the final compounds and the active site components of both the targets are discussed below.

**Binding Mode of HTS08531.** This compound was predicted with top GOLD fitness scores 83.582 and 68.077 for hLTA4H and hLTC4S, respectively. In terms of LTA4H binding, it has formed the hydrogen network with H295, H299, Y378, and Y383 as well as a coordinate bond with the metal ion. The nitrogen atom of the pyridine and oxygen

atoms of the oxadiazole ring have formed hydrogen bonds with H295 and H299, respectively, whereas one of the nitrogen atoms of the oxadiazole ring has formed hydrogen bonds with both H295 and H299 as well as coordinated with the metal ion. The carbonyl oxygen of the central amide has formed two hydrogen bonds with Y378 and Y383 residues. Hydrophobic interactions were mainly observed between the aromatic rings of the hit compound and amino acid residues such as F314, A377, M270, and Y267 (Figure 10b) (some of the residues are not shown in figure for clarity). At the active site of hLTC4S, it has formed hydrogen bonds with R31, R104, Y109, and W116 and also with the thiol group of GSH. The carbonyl oxygen of the central and terminal amide groups formed hydrogen bonds with Y109 and W116. Oxygen and one of the nitrogens present in the oxadiazole ring formed hydrogen bond interactions with R104 and the thiol group of GSH while the pyridyl nitrogen formed a hydrogen bond with R31. Considerable hydrophobic interactions were also observed between the ligand and active site residues (Figure 11b). The pharmacophore overlays of this compound upon LH-Hypo1 and LS-Hypo1 are shown in Figure 8a,c.



**Figure 11.** Binding poses of (a) compound 1 from the training set of hLTC4S, (b) HTS08531, and (c) KM09115, and (d) overlay of all three compounds in the active site of hLTC4S. Protein residues are shown in cyan (chain A) and pink (chain C) colored thin sticks. The glutathione (GSH) moiety is represented in the ball and stick mode. Hydrogen bonds are shown in red dashed lines.

**Table 5.** GOLD Fitness Scores and AutoDock Binding Energies of Final Possible Dual Inhibitors Identified in This Study

compound	LTA4H binding		LTC4S binding	
	GOLD fitness	binding energy (kcal/mol)	GOLD fitness	binding energy (kcal/mol)
HTS08531	83.582	−10.41	68.077	−6.45
HTS09460	83.308	−11.11	55.797	−7.57
KM09155	82.675	−11.67	64.388	−6.10
HTS02238	82.261	−11.32	54.827	−8.30
HTS13268	82.258	−12.79	61.430	−7.08
KM10378	82.103	−9.94	63.275	−6.33
HTS02824	81.327	−11.06	59.983	−7.65
SCR01204	80.543	−10.11	58.502	−6.93

**Binding Mode of KM09155.** The binding mode of this hit compound at the active site of hLTA4H formed a hydrogen bond network with the key amino acids H295, H299, Y378, and Y383. It has also formed a coordinate interaction with the metal ion. The carbonyl oxygen of the amide group has formed hydrogen bonds with H295 and H299 and a coordinate bond with the metal ion. The nitrogen atoms present in the central triazole ring interacted with Y378 and Y383 through hydrogen bonds. This compound has scored a GOLD fitness score value of 82.675 for hLTA4H binding (Figure 10c). This compound at the active site of hLTC4S has formed hydrogen bonds with key amino acids R104 and Y109 and the thiol group of GSH. One of the amide groups present in this hit compound and the sulfur atom formed the hydrogen bonds with the thiol group of GSH, R104, and Y109. Considerable hydrophobic interactions were observed from both the bindings (Figure 11c). The GOLD fitness score of this compound for hLTC4S binding is 64.388. The pharmacophore overlays of this compound upon LH-Hypo1 and LS-Hypo1 are shown in Figure 8b,d.

Molecular overlay of these two hits with the most active compounds in the corresponding training set at the active sites of hLTA4H and hLTC4S (Figures 10d and 11d).

**Binding Mode of HTS13268.** HTS13268 has scored the GOLD fitness scores of 82.258 for LTA4H binding and 61.43 for hLTC4S binding. Binding at the active site of hLTA4H revealed that the carbonyl oxygen atoms from pyrazine-2,5-dione and central amide moieties present in this compound formed hydrogen bonds with residues H295, H299, Y378, and Y383. It has also formed a coordinate bond with the metal ion (not shown in figure). hLTC4S binding of this compound showed hydrogen bond interactions with R104 and Y109. This compound has gained substantial hydrophobic interactions at both the active sites.

**Reverse Validation.** The druglike Maybridge database was first docked into the active site of hLTC4S, and 682 compounds scoring a GOLD fitness score value greater than any of the training set compounds were selected. These compounds were mapped over both hLTA4H and hLTC4S pharmacophore models. Fifty compounds have mapped all and any four of five pharmacophoric features of hLTA4H and hLTC4S pharmacophore models, respectively. Finally, these 50 compounds were docked into the active site of hLTA4H. Reverse validation procedure also has identified the same set of compounds along with two new compounds, namely, SCR01452 and KM10422. These compounds were excluded as they share the same chemical scaffolds with SCR01204 and KM09155. Chemical structures of the new compounds and docking results of reverse validation are provided in Tables S1 and S2 of the Supporting Information.



## CONCLUSION

In summary, a strategy combining molecular docking and pharmacophore filtering was applied to meet the critical challenges faced in designing efficient multitarget inhibitors to treat complex inflammatory diseases. Initially, a druglike database of small molecules was made and docked into one of the two targets (hLTA4H) using the widely accepted molecular docking program GOLD, and the compounds with better binding characteristics were selected for further study. Common feature pharmacophore models were developed for both the targets on the basis of the experimentally known inhibitors. Postprocessing of the docking results with pharmacophore filtering allowed us to bypass the difficulty with scoring functions that produce false positives from the molecular docking calculations. This pharmacophore filtering method ensured the selection of compounds with the required chemical features. The compounds filtered through both pharmacophore models were docked into the active site of the second target (hLTC4S). The compounds with better binding characteristics and with the required pharmacophoric features were selected as possible dual inhibitors for hLTA4H and hLTC4S enzymes to block the inflammatory signals through the AA pathway. Reverse validation also has suggested the same compounds as possible dual inhibitors. Further biological testing of hits would be necessary to absolutely determine the success rate of this work and optimize the hits subsequently.

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**Supporting Information Available:** Results of the reverse validation procedure along with the chemical structures of newly identified hits through this validation. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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