

Discovery of Novel 5-lox Inhibitors: Pharmacophore Design, Homology and Docking Studies

Sukesh Kalva, S Vadivelan, Sarma A. R. P. Jagarlapudi

Abstract—Inhibition of leukotriene biosynthesis has been extensively studied as a potential for the development of novel therapies for inflammation, respiratory diseases and, in particular, for asthma. We have designed specific functional inhibitors against 5-Lipoxygenase (5-LOX) using several virtual screening techniques like Homology modeling, MD simulations, Docking and Pharmacophore studies. We have identified 6 analogues of novel 5-LOX inhibitors ($IC_{50} < 19$ nM) using pharmacophore models and docking studies and their drug like properties were evaluated. These 6 compounds can be taken for further study including synthesis.

Index Terms—5-Lipoxygenase; Autodock; Docking; Molecular dynamics; Pharmacophore.

I. INTRODUCTION

5-LOX (5- Lipoxygenase) was found to be a cytosolic enzyme and contained a non-heme iron atom and calcium. This iron atom is critical to determine the catalytic potential of the enzyme, with oxidation to the ferric (Fe^{3+}) form conferring activity [1]. 5-LOX can be activated by an increase in intracellular Ca^{2+} concentration, diacylglycerols, phosphorylation by MAPKAP (mitogen-activated protein kinase-activated protein kinase) kinase-2 and ERK (Extracellular Receptor Kinase) [2]. In the absence of calcium, 5-LOX has minimal catalytic activity. The addition of calcium stimulates both the oxygenation and dehydration reactions of 5-LOX. This enzyme is expressed mainly in leukocytes, in line with the function of leukotrienes as mediators of immune reactions [3]. Granulocytes, monocytes/macrophages, mast cells, dendritic cells and B lymphocytes express 5-LOX, whereas platelets, endothelial cells, T cells and erythrocytes do not. 5-lipoxygenase (5-LOX) catalyzes the first two steps in the synthesis of all LTs (Leukotriene's), namely the oxidation of AA to 5-hydroperoxyicosatetraenoic acid (5HPETE) followed by the dehydration of 5-HPETE to LTA [4]. 5-Lipoxygenase has a central role in the biosynthesis of leukotrienes.

This enzyme initializes the formation of proinflammatory leukotrienes from arachidonic acid (AA). The potent biological effects of Leukotriene's (LTs) includes leukocyte

aggregation, smooth muscle contraction and vascular permeability which mimic the biological changes associated with the patho-physiology of inflammatory disorders such as asthma, rheumatoid arthritis, inflammatory bowel disease and psoriasis [5]. Recently, the 5-LOX pathway has also been associated with atherosclerosis [6], osteoporosis [7] and certain types of cancer like neuroblastoma [8] and prostate cancer [9]. Increased activity of the 5-LOX pathway, which includes another protein termed FLAP (5-lipoxygenase-activating protein), is strongly associated with cardiovascular disease (CVD) risks, including that for stroke [9]. In addition to its presence in the CVD (Cardiovascular diseases), 5-LOX is expressed in the brain [10], where it's functioning may be independent of cardiovascular activity. In the brain, 5-LOX participates in the regulation of neurotransmitter receptors, e.g., glutamate [5]. Inhibition of 5-LOX will inhibit the formation of LTB₄ as well as that of the cysteinyl leukotrienes (LTC₄, LTD₄ and LTE₄). Many 5-LOX inhibitors have subsequently been developed, and several have displayed efficacy in asthma models, such as allergen-induced bronchoconstriction [11]. In the present study, the molecular modeling, pharmacophore and structure based drug designing studies were done using MODELLER 9v7, Catalyst and Autodock 4.0 to design the specific functional inhibitors that target the selective amino acids within the modeled enzyme.

II. MATERIALS AND METHODS

A. Database mining and training set

Quantitative pharmacophore hypotheses were generated using a set of 102 5-LOX inhibitors based on the diversity of chemical structure and biological activity against human 5-LOX inhibitors. The most critical aspect of pharmacophore hypothesis generation is the selection of the training set. A set of 102 5-LOX inhibitors from 60 references (inclusive of journals and patents) with an activity range (IC_{50}) i.e., 9 - 50000 nM was selected [12]. This group was divided into training and test sets. The training set of 22 molecules was designed to be structurally diverse with a wide activity range. The test set of remaining 80 molecules is designed to evaluate predictive ability of the resultant pharmacophore. Some of the molecules selected as the training set are given in Fig. 1 This training set is used in the HypoGen to generate the quantitative pharmacophore models [13].

B. HypoGen

For generating HypoGen model, a minimum number of features involving hydrogen bond acceptor, hydrogen bond

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donor and hydrophobic have been specified based on the 5-LOX inhibitor activities. The molecules in the training set are broadly classified into three categories namely, highly active (<10-200 nM), moderately active (200-1000 nM) and low active (>1000 nM) while an uncertainty value of 3 was used. The quality of HypoGen models are best described in terms of Fixed Cost, Null Cost and Total Cost and these terms are well defined by Debnath [14]. For a good model, the total cost of any hypothesis should be close to the fixed cost. The best model Hypo-1 has been given in Fig. 2 and the parameters that describe Hypo-1 are given in Table I.

C. Homology modeling for 5-LOX

The homology modeling of 5-LOX was performed using MODELLER 9v7 [15]. The amino acid sequence of human 5-LOX was retrieved from SWISS PROT (No. P09917) [16]. Template search for 5-LOX was carried out using the MODELLER 9v7 software and the crystal structure of soyabean lipoxygenase-3 (PDB code: 1IK3) was obtained from PDB database, and it shares 32% sequence identity with the human 5-LOX. It is indeed well known that a homology-modeled structure of a target protein can be accurate enough to be used in docking studies, once the sequence identity between target and template approaches 40% [17]. To further equilibrate, the 3D model was subjected to a short molecular dynamics (MD) refinement. MD calculations were carried out using the GROMACS [18] program package. The best homology model was relaxed for 200 picoseconds in order to obtain equilibrium.

D. ADME properties

The above 6 ligands were submitted to the MolSoft server to check the drug like properties like Molecular weight, Log P, Hydrogen bond donor, Hydrogen bond acceptor to qualify the drug characteristics of the above compounds (Table IV).

E. Docking studies

Autodock is able to predict the protein-ligand complex structures with reasonable accuracy and speed [19]. To identify the binding sites of the protein with the ligand, blind docking was performed using Autodock. AutoDock can be used when the location of the binding site is unknown. [20-21]. The docking was applied to the whole protein target, with a grid covering the whole surface of the protein. The ligand models were docked to the modeled 5-LOX receptor using the default settings of Lamarckian genetic algorithm. Hence, a flexible ligand was docked to a rigid protein. The scoring functions in the docking software are based on interpolated energy evaluation. The results of the docking studies were 10 best docked ligands quantified in terms of the calculated free energy of binding (ΔG).

III. RESULTS AND DISCUSSION

A. Pharmacophore generation and validation studies

In the HypoGen studies, a couple of sets of 10 hypotheses were generated using the most diverse 22 molecules in the training set. The best hypothesis consists of 1) two hydrogen bond acceptors, 2) two hydrophobic groups. The values of ten hypothesis such as cost, correlation (r),

and root-mean-square deviations (RMSD) are statistically significant (Table I). It is evident that as error, weight and configuration components are very low and not deterministic to the model, the total pharmacophore cost is also low and close to the fixed cost. Also, as total cost is less than null cost, this model accounts for all the pharmacophore features and has a good predictive ability. Two statistical methods were employed to rank the ten resultant hypotheses. In the first method, all the ten hypotheses were evaluated using a test set of 80 known 5-LOX inhibitors, which are not included in the training set. Predicted activities of the test set were calculated using all ten hypotheses and correlated with the experimental activities. Of the ten hypotheses, Hypo-1 showed a better correlation coefficient (0.891) compared to the other nine hypotheses.

A second statistical test includes calculation of false positives, false negatives, enrichment and goodness of hit to determine the robustness of hypotheses. Under all validation conditions, Hypo-1 performed superior as compared to the other hypotheses. Hypo-1 demonstrated excellent prediction of 5-LOX inhibitory activities of the training set compounds (Table III). Analyzing the results, it was observed that out of 8 highly active molecules, 5 were predicted correctly as highly active, and the rest were predicted as moderately active. Among the 10 moderately active molecules, 9 molecules were predicted as moderately active, 1 was predicted as highly active. Out of 4 low active molecules, all of them were predicted as low active. Activities of the compounds were not correctly predicted, but the fit values also confer a good measure of how well the pharmacophoric features of Hypo-1 were mapped onto the chemical features of the compounds. Fig. 2 shows the Three-dimensional arrangement of pharmacophore features in the quantitative pharmacophore model (Hypo-1). Fig. 2A shows the all the features of Hypo-1 (acceptor and Hydrophobic) were mapped onto the highly active compound of training set (1). Fig. 2B shows the all the features of Hypo-1 were partially mapped onto the inactive compound of training set (22).

The correlation values along with the above predictions make the pharmacophore suitable to predict molecular properties well. The plot showing the correlation between the actual and predicted activities of the test and training set molecules is given in Fig. 4. This indicates that the pharmacophore model generated is capable of predicting the activity of the unknown molecules with the reasonable accuracy. Hypo-1 was used to search the test set of known 5-LOX inhibitors. Database mining was performed using the BEST flexible searching technique. The results were analyzed using a set of parameters such as hit list (Ht), number of active percent of yields (%Y), percent ratio of actives in the hit list (%A), enrichment factor of 2.91 (E), false negatives, false positives, and goodness of hit score of 0.75 (GH) (Table II) [22]. Hypo-1 succeeded in the retrieval of 80% of the active compounds. In addition, the pharmacophore also retrieved 6 inactive compounds (false positives). It predicted 4 active compounds as inactive (false negatives). An enrichment factor of 2.91 and a GH score of 0.75 indicating the quality of the model are acceptable.

Overall, a strong correlation was observed between the Hypo-1 predicted activity and the experimental 5-LOX inhibitory (IC_{50}) of the training and test compounds (Fig. 4).

However, the Hypo-1 model has a greater tendency to show false positives. This could be attributed to high structural similarity in the active and inactive 5-LOX inhibitors, resulting in an inability to discriminate this pattern by the pharmacophore model. We have selected Hypo1 as a 3D query to search a subset of GVK BIO in-house database of ~1 M compounds to retrieve compounds with novel structural scaffolds and desired features [23]. The initial screening of Hypo1 yielded 3000 compounds and further cluster analysis of these hits corresponded to 220 unique cluster representatives. We further extended this study to structure-based design and to limit the number of false positive and false negative hits and to further understand the binding of inhibitors to the active site of 5-LOX complex. Fig. 5 shows some of the identified and optimized potent lead molecules through virtual library screening.

B. 5-LOX model

The 3d structure of the target protein was built by homology modeling based on the template protein. The best modeled structure was taken based on the DOPE score among the five modeled structures; it was found that the model1 shows highest DOPE score. The modeled protein is minimized using GROMACS 4.0. The protein was position restrained up to 200 picoseconds. The simulation was performed up to 1 nanosecond. The average structure was taken from simulation. The model was further checked by WHAT IF and Procheck [24], the final 5-LOX model (after equilibration and re-minimization) shows that, more than 85.1% of the residues are in the core regions of the Ramachandran plot. The final 5-LOX model was subjected for docking studies. As per the results of Chimera the root mean square deviation (RMSD) value was found to be 0.472 Å. Comparison of sequences between 1IK3A and 5-LOX using ClustalW (Fig 3). Superimposition of the model over the template 1IK3 was done in Chimera. As per the results of Chimera the root mean square deviation (RMSD) value was found to be 0.472 Å.

C. Docking studies

A set of 5-LOX inhibitors was docked to modeled 5-LOX protein using AutoDock. Overall, there was not much significant correlation between the fitness scores and 5-LOX inhibitory activities of the compounds. Dock log file generated from the Autodock shows the ligand docked to the 5-LOX with the least binding energy -6.05 (kcal/mol) and shows rank 1 in the clustering histogram table. Hydroxyl groups of the ligand show hydrogen bonding with the His 368. The complex is also further stabilized with the NH interaction with the Gln 364. Fig 6 shows the ligand bound inside a pocket in 5-LOX. This analysis concludes the important interaction between the 5-LOX protein and ligand and also correlated these interactions with pharmacophore features. Drug like properties were

calculated for the 6 compounds.

IV. CONCLUSIONS

The best quantitative Pharmacophore model for all hypotheses had in common two hydrogen bond acceptors and two hydrophobic groups indicating the importance of these groups in making the molecule, a potent 5-LOX inhibitor. The Pharmacophore models were validated using the test set prediction and Database screening. The most active molecule in the training set fits very well with the top scoring Pharmacophore hypothesis. The generated Pharmacophore model from the 5-LOX inhibitors can be used as a three dimensional query in database searches to identify novel compounds with diverse structures that can function as a potent inhibitor and to evaluate how well the newly designed compounds maps on the Pharmacophore before undertaking any further study including synthesis.

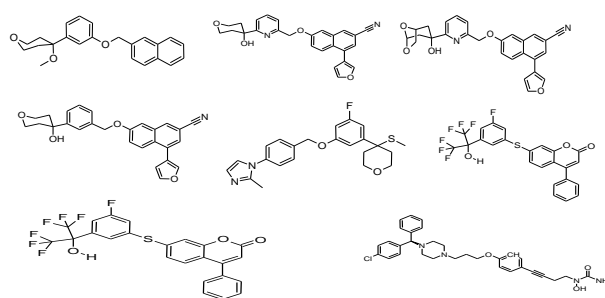


Fig. 1. Structures of some training set molecules.

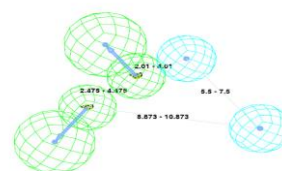


Fig 2. HypoGen feature with its distance constraints, features are color coded with green: hydrogen bond acceptor and light blue: hydrophobic

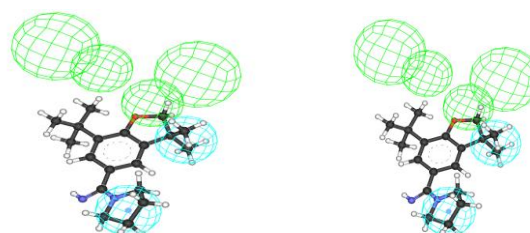


Fig. 2A. Hypo- 1 mapped to a highly active compound (1)

Fig. 2B. Hypo-1 mapped to a low active compound (22)

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5-1ox
1IK3_A|PDBID|CHAIN|SEQUENCE      -----MPSYTVTVATG 11
                                     MLGGLLHRGHKIKGTVVLMRKNVLDVNSVTSVGGIIGQLDLVGSSTLDTL 50
                                     : * :
5-1ox
1IK3_A|PDBID|CHAIN|SEQUENCE      SQWFAGTDDYIYLSLVGSAGCSEKHLLDKPFYNDFER-----GAVDSYF 55
1IK3_A|PDBID|CHAIN|SEQUENCE      TAFLGRSVSLQLISATKADANGKGLGKATFLEGIITSLPTLGAGQSAFD 100
                                     : : : : : * : : : * : : :
5-1ox
1IK3_A|PDBID|CHAIN|SEQUENCE      VTVDEELGEIQLVRIEKRYWLNDDWYLKYITLKTPHGDYIEFPCYRWIT 105
1IK3_A|PDBID|CHAIN|SEQUENCE      INFVDDGSGIPGAFYIKNFMQTEFFLVSLTLEDIPNHGSIHFVCSNWIY 150
    
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5-lox      :... : * .      : : : : . : : : .      . * : . * * * * *
1IK3_A | PDBID | CHAIN | SEQUENCE  -----GDVEVVLRDGRAKLAR--DDQIHLKQHRRKELETRQK 141
NAKLFKSDRIFFANQTYLPSETPAPLVKYREELHNLRGDGTGERKEWER 200
      . :      : :      : * * . : : : * * : : : * * : : :
5-lox      QYRWMEWNP-----FPLSIDAKCHKD----- 163
1IK3_A | PDBID | CHAIN | SEQUENCE  IYDYDVYNDLGDPPDKGENHARVPLGGNDTFYPYRRGRTRGKPTRKDPNSE 250
      * : : * *      * : * *      * * . : : . :
5-lox      -----LPRDIQFDSEKGVDFVLNYSKAMENLFINRFMHMFQSSWN--D 204
1IK3_A | PDBID | CHAIN | SEQUENCE  SRSNDVYLPDRDEAFGLHKSSDFLTYGLKSVSQNVLPQLQSADFNLNFTPRE 300
      * * * * * * . * . * * : * : : : . : : * : . : . :
5-lox      FADFEKIFVKISN-----TI 219
1IK3_A | PDBID | CHAIN | SEQUENCE  FDSFDEVHGLYSGGKILPTDIISKISPLPVLKEIFRTDGEQALKFPPPKV 350
      * * : : : . : * .      :
5-lox      SERVMNHWQEDLMFGYQFLNGCNPVLIIRCTELP-----EKLK 257
1IK3_A | PDBID | CHAIN | SEQUENCE  IQVSKSAWMTDEEFAREMLAGVNPNIIRCLKDFPPRSKLDQSVYGDHTSQ 400
      : . * * . : * * * * * * * * * * * * * * * * * * * * *
5-lox      VTTEMVESLRLQSLQEQVQGNIFIVDFELLDGIDANKTDPCTLQFLA 307
1IK3_A | PDBID | CHAIN | SEQUENCE  ITKEHLEPNLEGLTVDIAIQNKRLFLLDHHDPIMPYLRRINATSTKAYA 449
      : * * * * * * * * * * : : : : * : : * * . : : : : *
5-lox      APICLLYKN-LANKIVPIAIQLNQIPGDEN----PIFLPSDAKYD--WL 349
1IK3_A | PDBID | CHAIN | SEQUENCE  TRTILFLKNDGTLRPLAIELSLPHQGDQSGAFSQVFLPADEGVSESIWL 499
      : * : * * : : : * : * : * * : * * : * * * * * * * *
5-lox      LAKIWRSSDFHVHQTITHLLRTHLVSEVFGIAMYRQLPAVHPFIFKLLVA 399
1IK3_A | PDBID | CHAIN | SEQUENCE  LAKAYVVVNDSCYHQLVSHWLNTHAVVEPFIIATNRHLSVHPIYKLLHP 549
      * * * * * * * * * * * * * * * * * * * * * * * * * * * *
5-lox      HVRFTIAINTKAREQLICEGLFDKANATGGGGHVQMVQRAMKDLTYASL 449
1IK3_A | PDBID | CHAIN | SEQUENCE  HYRDTMNINGLARLSLVNDGGVIEQTFWLG-RYSVEMSAVVYKDWVETDQ 598
      * * * * * * * * * * * * * * * * * * * * * * * * * * *
5-lox      CFPEAIKARGMESKED-----IPYFYRDDGLLVWEAIRTFTEA 490
1IK3_A | PDBID | CHAIN | SEQUENCE  ALPADLIKRGMAIEDPSCPFGIRLVIEDPYTVDGLIWDIAIKTWVHEYV 648
      : * : * * * * * * * * * * * * * * * * * * * * * * * *
5-lox      DIYEGDQVVEEDPELQDFVNDVYVGMGRKSSGFPKSVKREQLSEYL 540
1IK3_A | PDBID | CHAIN | SEQUENCE  FLYYKSDDTLREDPELQACWKEVVEVGHGDKKNEPWWPKMQTRFEEV 698
      : * * : * : : * * * * * * * * * * * * * * * * * * * *
5-lox      TVVIFTASAQHAAVNFGQYDWCWIPNAPPTMRAPP-----TAKGVV 583
1IK3_A | PDBID | CHAIN | SEQUENCE  AIIIWTSALHAAVNFGQYPYGLILNRPPLSRFRMPEKGSABEYELRKN 748
      : : * * * * * * * * * * * * * * * * * * * * * * * *
5-lox      TIEQIVDTLPDRGRSCWHLGAVWALSQFQENELFLGMYPEEHFIEKP-VK 632
1IK3_A | PDBID | CHAIN | SEQUENCE  PQKAYLKTITPKFQTLIDLSVIEILSRHASDEVYLGERRDNPNTSDTRAL 798
      : : * * . : : : : * : : : * * : * * : * * : : : . :
5-lox      EAMARFRKNLEAIVSVIAERNKKK-----QLPYYYLSPDR----- 667
1IK3_A | PDBID | CHAIN | SEQUENCE  EAFKRFGNKLQAIENKLSERNNDEKLRNRCGPVQMPYTLPLPSSKEGLTF 848
      * * : * * : * * * . : * * * * * : * * * * * * *
5-lox      --IPNSVAI 674
1IK3_A | PDBID | CHAIN | SEQUENCE  RGIPNSISI 85
      * * * * :
    
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Fig 3. Sequence alignment of the query, 5-LOX with the template, 1IK3, soybean lipoxygenase-3 using clustalW. Identical residues are denoted by asterisk (*).

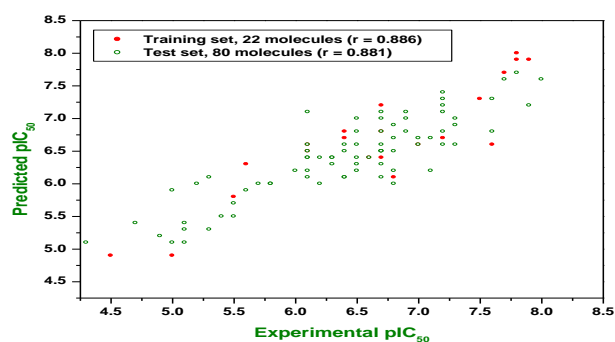


Fig 4. Graph showing the correlation between experimental and predicted activities for the 5-LOX inhibitors (22 molecules in training set and 80 molecules in the test set) against comparative model.

Molecule	Predicted IC ₅₀	Molecule	Predicted IC ₅₀
	2.8 nM		14 nM
	5.8 nM		16 nM
	6.1 nM		19 nM

Fig 5. Some of the identified and optimized potent lead molecules through virtual screening

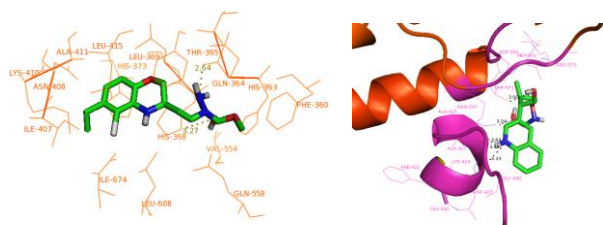


Fig 6. Inhibitor and residues within 4 Å around the active site are denoted as sticks. H-bonds are shown in magenta color. Amino acid residues are represented as lines. Ligand is represented as ball and sticks.

TABLE I. 10 PHARMACOPHORE MODELS GENERATED BY THE HYPOGEN ALGORITHM

Hypo No	Total Cost	Cost difference ^a	Error Cost	RMS deviation	Training set (r)	Features ^b
1	102.95	9.192	81.85	0.844	0.891	AAHH
2	103.80	8.34	82.53	0.88	0.881	AAHH
3	104.27	7.869	83.14	0.911	0.872	AAHH
4	104.40	7.737	83.22	0.915	0.871	AAHH
5	104.50	7.639	82.81	0.895	0.877	AAHH
6	106.35	5.788	85.01	1.002	0.843	AAHH
7	106.43	5.708	84.83	0.992	0.847	AAHH
8	106.85	5.296	84.99	0.999	0.845	AAHH
9	108.07	4.076	86.81	1.079	0.815	AAHH
10	108.20	3.944	86.70	1.074	0.817	AAHH

^a (Null cost-Total cost), Null cost = 112.14, Fixed cost = 95.05, For the Hypo-1 Weight=1.16 Config=15.93. All cost units are in bits. ^b A- Hydrogen Bond Acceptor, H – Hydrophobic.

TABLE II. VALIDATION OF THE PHARMACOPHORE MODEL USING STATISTICAL PARAMETERS

Total compounds in database (D)	102
Total Number of actives in database (A)	28
Total Hits (Ht)	30
Active Hits (Ha)	24
% Yield of Actives	80.00
% Ratio of actives in the Hit list	85.71
Enrichment factor or Enhancement (E)	2.91
False Negatives	4
False Positives	6
GH score (Goodness of Hit list)	0.75

[[((Ha/4HtA) (3A +Ht))_1_((Ht_Ha)/(D_A))]]; GH score of 0.6–0.7 indicates a good model

TABLE III. EXPERIMENTAL AND PREDICTED IC₅₀ DATA OF 22 TRAINING SET MOLECULES

Molecule	Fit value	Experimental ¹ IC ₅₀ , nM	Predicted IC ₅₀ , nM	Error ^a	Experimental Scale	Predicted Scale
1	6.82	14	13	-1.1	+++	+++
2	6.87	15	11	-1.3	+++	+++
3	6.8	17	13	-1.3	+++	+++
4	6.62	20	20	-1	+++	+++
5	5.53	26	240	9.3	+++	++
6	6.17	30	56	1.9	+++	+++
7	5.67	70	180	2.5	+++	++
8	5.54	90	240	2.7	+++	++
9	5.04	150	750	5	++	++
10	6.14	180	60	-2.9	++	+++
11	5.72	190	160	-1.2	++	++
12	5.29	200	430	2.1	++	++
13	5.31	230	400	1.8	++	++
14	5.32	270	400	1.5	++	++
15	5.65	370	190	-2	++	++
16	5.72	400	160	-2.6	++	++
17	5.46	740	290	-2.6	++	++
18	5.51	810	260	-3.1	++	++
19	5.18	2300	540	-4.2	+	+
20	4.68	3000	1700	-1.7	+	+
21	3.81	9000	13000	1.4	+	+
22	3.82	30000	13000	-2.4	+	+

^a + indicates that the predicted IC₅₀ is higher than the experimental IC₅₀; - indicates that the predicted IC₅₀ is lower than the experimental IC₅₀; a value of 1 indicates that the predicted IC₅₀ is equal to the experimental IC₅₀. ^b Fit value indicates how well the features in the pharmacophore overlap with the chemical features in the molecule. Fit = weight × [max (0.1–SSE)] where SSE = (D/T) 2, D = displacement of the feature from the center of the location constraint.

TABLE IV. THE LIGANDS CHECKED FOR THE DRUG LIKE PROPERTIES WITH LIPINSKI'S RULE OF FIVE

Ligand	Molecular weight	HBA ¹	HBD ²	LogP ³
66899	387.1	7	1	0.76
34652	328.16	5	3	0.93
66883	381.17	7	1	1.07
17847	338.12	4	2	3.36
34665	336.13	5	3	2.24

- 1) HBA: Hydrogen bond acceptor,
- 2) HBD: Hydrogen bond donor,
- 3) Log P: Octanol-water partition coefficient

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