P56 LCK Inhibitor Identification by Pharmacophore Modelling and Molecular Docking

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Pharmacophore models for lymphocyte-specific protein tyrosine kinase (P56 LCK) were developed using *CATALYST HypoGen* with a training set comprising of 25 different P56 LCK inhibitors. The best quantitative pharmacophore hypothesis comprises of one hydrogen bond acceptor, one hydrogen bond donor, one hydrophobic aliphatic and one ring aromatic features with correlation coefficient of 0.941, root mean square deviation (RMSD) of 0.933 and cost difference (null cost-total cost) of 66.23. The pharmacophore model was validated by two methods and the validated model was further used to search databases for new compounds with good estimated LCK inhibitory activity. These compounds were evaluated for their binding properties at the active site by molecular docking studies using *GOLD* software. The compounds with good estimated activity and docking scores were evaluated for physiological properties based on Lipinski's rules. Finally 68 compounds satisfied all the properties required to be a successful inhibitor candidate.

Key Words : P56 LCK, Protein tyrosine kinase (PTKs), *CATALYST* pharmacophore hypothesis, Molecular docking, Lipinski's rules

Introduction

Eukaryotic protein kinases constitute a large family of homologous proteins that catalyze the transfer of the gamma phosphate group of ATP or GTP to the hydroxyl group of serine, threonine or tyrosine in a substrate protein.[1] Protein tyrosine kinases (PTKs) are critically involved in signaling pathways that regulate cell growth, differentiation, activation, and transformation.[2-4] Malfunctions of cellular signaling have been associated with many diseases including cancer and diabetes. PTKs can be divided into receptor tyrosine kinases (RTKs) and non-receptor (cytosolic) tyrosine kinases.[5,6] Non-receptor tyrosine kinases belonging to the Src family are key players in signal transduction. Some Src kinases (Fyn, Src, Yes) are found in most cell types whereas others exhibit a more restricted tissue distribution (Lck, Hck, Blk) and have more specific tasks in signal transduction.[7]

Lymphocyte-specific protein tyrosine kinase (LCK) is a member of the Src family of non-receptor protein tyrosine kinases,[8,9] expressed primarily in T-Lymphocytes and natural killer cells.[10,11] LCK is essential for T-cell development and function.[12] It is constitutively associated with the cytoplasmic portions of the CD4 and CD8 surface receptors and plays a key role in T-cell antigen receptor (TCR) linked signal transduction pathways.[13-15] Studies have shown that the catalytic activity of LCK is regulated by tyrosine phosphorylation at two sites: Tyr394 in the catalytic domain and Tyr505 on the C-terminus.[16] When Tyr394 is phosphorylated and Tyr505 is dephosphorylated, the fully activated enzyme can phosphorylate tyrosine residues within a special sequence called the immunoreceptor tyrosine activation motif (ITAM) located on the ζ-chain of the TCR. This phosphorylation creates a docking site for its downstream substrate ZAP-70. Subsequent phosphorylation of ZAP-70 by LCK[17,18] triggers a series of downstream cascade events that lead to mobilization of intracellular calcium ion[19] and activation of protein kinase C (PKC), a serine/threonine specific kinase.[20,21] Inhibitors of LCK may have potential therapeutic ability in the treatment of auto-immune diseases such as coxsackievirus B3-mediated heart diseases, rheumatoid arthritis, multiple sclerosis, lupus, as well as inflammatory diseases, prevention of solid organ transplantation and allergic diseases.[22-24] There have been several reports of inhibitors of LCK. Much of the earlier work was on natural products and compounds derived from them related to the nonselective tyrosine kinase inhibitors. Recently other inhibitors have been derived from the tyrphostins, quinazolines, pyrazolopyrimidine PP1, thiazole, benzothiazole, pyrido[2,3-d]pyridine inhibitors, which have potent activity against LCK.

The main aim of this study is to construct a pharmacophore model based on key chemical features of compounds with LCK inhibitory activity and then is to find the new lead candidate molecules through database searching using the pharmacophore model. In our earlier studies, the key chemical groups of benzothiazole analogs were extensively studied by using quantitative structure-activity relationships (QSAR) analysis methods and their binding modes were established by molecular docking studies.[25] Unlike the previous QSAR study, which is based on single scaffold containing molecules, here we have collected compounds from the various scaffolds and have generated pharmacophore model that can provide a rational hypothetical picture of the primary chemical features responsible for activity. The best pharmacophore model is expected to provide useful knowledge for

developing new potentially active candidates targeting the P56 LCK.

Methods

Pharmacophore Model Development and Validation. Pharmacophore models were developed using a data set of different inhibitors for P56 LCK by using the *CATALYST* 4.10 *HypoGen* module as there is, so far, no report on developing pharmacophore models using inhibitors for P56 LCK. The *CATALYST* program,[26] one of the leading automated drug design software was mainly used for the study since a large number of successful applications were clearly demonstrated in medicinal chemistry.[27,28] This study is expected to provide useful knowledge for developing new inhibitors targeted to P56 LCK activity. The most important aspect of the hypothesis generation is the selection of the training set molecules. The training set must have structural diversity and wide coverage of activity range (4-6 orders of magnitude). To accomplish such a task we have collected and developed P56 LCK inhibitor database with biological activity data from various medicinal chemistry as well as life science journals using *MDL ISIS/Base*.[29] Thus our LCK inhibitor in-house database comprises of 534 compounds with experimental activities.

Following the basic principles of training set selection, 25 compounds[30-39] were exquisitely chosen with their structures shown in Figure 1. Training and test set compounds taken from our in-house database were imported into *CATALYST*, and submitted for conformational analysis (max. number of conformers 250, generation type: best quality, energy range 20 kcal/mol above the local minimized structure). Training set compounds were used for generating pharmacophore models. The resultant pharmacophore models revealed that the four chemical feature types such as hydrogen bond acceptor (HBA), hydrogen bond donor (HBD), hydrophobic aliphatic (HY-ALI) and ring aromatic (RA) features could effectively map all critical chemical features of all active molecules in the training and test sets.

The purpose of the pharmacophore hypothesis generation is not just to predict the activity of the training set compounds accurately but also to verify whether the pharmacophore models are capable of predicting the activity for any given compounds and classifying them correctly as active or

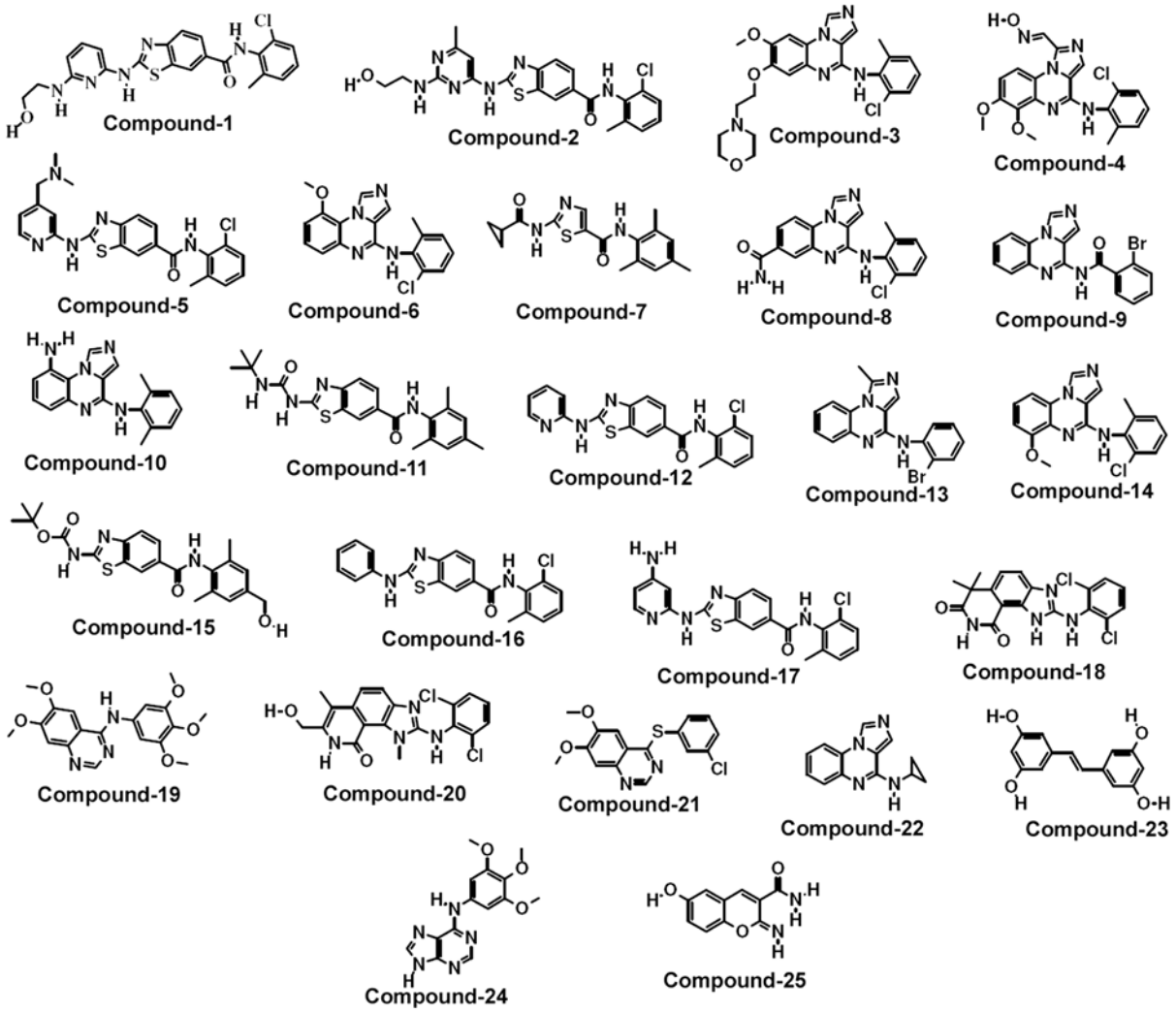

Figure 1. Molecular structures of 25 training set compounds.

inactive. In order to validate our pharmacophore hypothesis, we have used a test set comprising of 178 molecules which had experimental P56 LCK inhibitory activity belonging to different activity ranges and structural classes (data not shown). It was further cross validated by *Cat-Scramble* program available in *CATALYST*, which is based on Fischer's randomization test. The procedure of this technique is to randomize the activity data associated with the training set compounds, and the randomized training sets are used to generate pharmacophore hypotheses using the same features and parameters as developed for the original pharmacophore hypothesis. If the randomized data set results in the generation of a pharmacophore with similar or better cost values, RMSD, and correlation, then the original hypothesis is considered to have been generated by chance.

Database Search for New Hits. The best ranked four featured pharmacophore model, Hypo1 was used as a search query to retrieve molecules with novel and desired chemical features from multi-conformational NCI chemical database consisting of 238,819 compounds available in *CATALYST* software. The *Best Flexible Search Databases/Spread Sheets* method in *CATALYST* was used to search the database for similar featured compounds.

Molecular Docking Analyses. Biomolecular interactions and binding properties were analyzed for new hits that were obtained in the database search by using *GOLD* molecular docking. The program *GOLD* 3.0 (Genetic Optimisation for Ligand Docking) from Cambridge Crystallographic Data Center, UK,[40] uses genetic algorithm for docking flexible ligands into protein binding sites. The ATP binding site, which was filled with ANP (phosphoaminophosphonic acidadenlate ester) in the X-ray structure (PDBID: 1QPC), was used to define active site region. Active site radius was taken as 10.0 Å around ANP molecule. The annealing parameter of van der Waals interaction and hydrogen bond interaction were 4.0 Å and 2.5 Å respectively.

Drug-like Property Calculation. Lipinski's rule-of-five is a simple model to forecast the absorption and intestinal permeability of a compound.[41] According to the rule, compounds are considered likely to be well-absorbed when they possess LogP less than 5, molecular weight less than 500, number of H-bond donors less than 5, number H-bond acceptors less than 10, and number of rotatable bonds less than 10. All these properties were calculated using *Molinspiration* online database.[42-43]

Results and Discussion

A meaningful pharmacophore hypothesis may result when the difference between null and fixed cost value is large. The total cost of any pharmacophore hypothesis should be close to the fixed cost. The best significant pharmacophore hypothesis should be characterized by the high cost difference, low root mean square deviation (RMSD) and must have the best correlation coefficient. In our study, all the ten hypotheses have the same features: HBA, HBD, HY-ALI and RA. The cost values, correlation coefficients (r) for training and test sets, RMSD, and pharmacophore features are listed in Table 1. The null cost and the fixed cost value of the ten best ranking hypotheses are 178.9, and 100.775 respectively. Configuration cost, a constant value preferably less than 17, describing the complexity of the hypotheses space to explore, is 15.56. The first hypothesis Hypo1 as shown in Figure 2a, is characterized by highest cost difference (66.23), lowest RMSD value (0.933) and also with best correlation coefficient value (0.941), which represents a true correlation with good predictivity and henceforth has been suggested as the best pharmacophore hypothesis.

The experimental and estimated activities by the best pharmacophore hypothesis (Hypo1) for 25 training set compounds are shown in Table 2. Training set compounds were classified relatively into three sets based on their activity values: highly active (+++, IC50 ≤ 100 nM); moderately active (++, 1000 nM > IC50 > 100 nM) and inactive (+, ≥ 1000 nM). All highly active compounds (+++) were estimated correspondingly, four moderately active (++) compounds were estimated as highly active (+++) and all inactive molecules were estimated as inactive by Hypo1. Thus Hypo1 was able to estimate the activities of molecules in their own activity ranges. Compound 1, which is highly

Table 1. Information of statistical significance and predictive power presented in cost values measured in bits for top-ten hypotheses[a]

Hypothesis	Total cost	Cost difference (null-total cost)	RMSD	Correlation	Features	Correlation value for 178 test set compounds
1	112.675	66.225	0.933	0.941	HBA, HBD, HY-ALI, RA	0.8768
2	116.066	62.834	1.065	0.922	HBA, HBD, HY-ALI, RA	0.8081
3	116.682	62.218	1.106	0.916	HBA, HBD, HY-ALI, RA	0.8470
4	117.340	61.560	1.105	0.916	HBA, HBD, HY-ALI, RA	0.8470
5	118.212	60.688	1.159	0.907	HBA, HBD, HY-ALI, RA	0.8300
6	118.819	60.081	1.127	0.914	HBA, HBD, HY-ALI, RA	0.8532
7	119.209	59.691	1.201	0.900	HBA, HBD, HY-ALI, RA	0.8231
8	119.732	59.168	1.225	0.895	HBA, HBD, HY-ALI, RA	0.8337
9	119.977	58.923	1.234	0.894	HBA, HBD, HY-ALI, RA	0.8398
10	120.035	58.865	1.210	0.898	HBA, HBD, HY-ALI, RA	0.8170

[a]Null cost of top-ten score hypotheses is 178.9 bits Fixed cost is 100.775 bits. Configuration cost is 15.56 bits. Abbreviation used for features: HBA (hydrogen-bond acceptor), HBD (hydrogen-bond donor); HY-ALI (hydrophobic aliphatic); RA (ring aromatic).

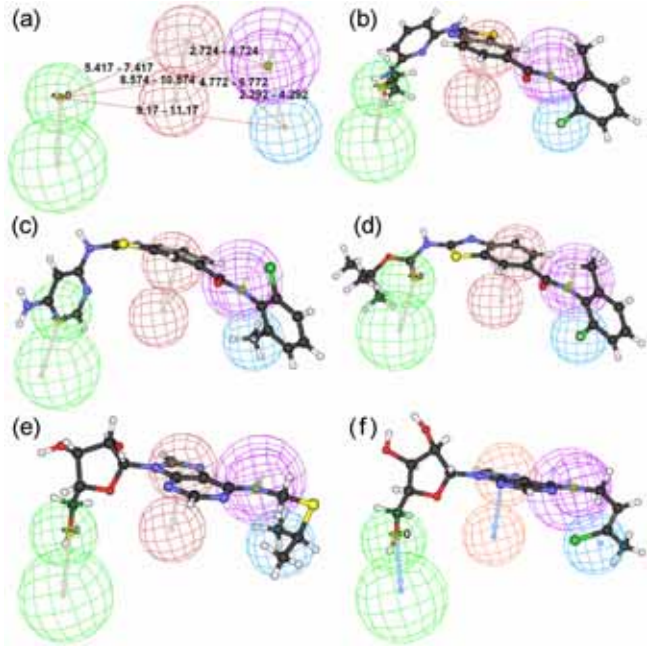

Figure 2. Pharmacophore hypothesis with distance geometry (a), pharmacophore hypothesis mapping on compound 1 of training set (b), T24 of test set (c), T35 of test set (d), new compound NCI0310811 (e) and NCI0303873 (f). In hypothesis green represents H-bond acceptor (HBA), orange represents ring aromatic (RA), magenta represents H-bond donor (HBD) and light blue represents hydrophobic aliphatic (HY-ALI).

active compound in the training set has been mapped perfectly by Hypo1 with a fit value of 9.28 as shown in Figure 2b and was able to estimate its activity accurately as 0.5 (experimental activity IC50 = 0.5 nM).

All molecules in the test set were built and conformational analysis was done similar to that of training set. Among 178 test set compounds, 173 compounds had an error value < 10, representing a not more than one order difference between estimated and experimental activity. The 0.876 correlation coefficient achieved for the test set by using Hypo1 shows a good correlation between experimental and estimated activity. Correlation coefficient values for all 10 pharmacophores are shown in Table 1. The correlation graph between experimental and estimated activities was shown in Figure 3. The mapping of validated model, Hypo1 on two compounds in the test set, compound T24 and T35 (IC50 = 8 and 70 nM respectively), are represented in Figure 2c and Figure 2d respectively. All the Hypo1 features overlapped with the chemical groups of these two compounds and thus this model accurately estimated the IC50 values as 10 and 71 nM respectively.

Another method to validate the quality of *HypoGen* hypothesis is to apply cross validation using the *Cat-Scramble* program. The goal of this type of validation is to check whether there is a strong correlation between the chemical structures and the biological activity. The 19 spreadsheets were generated with 95% confidence level and the results of

Table 2. Experimental activity and estimated activity of training set molecules based on pharmacophore model Hypo1

Compound No.	Experimental Activity (nM)	Estimated Activity	Error[a]	Fit Value[b]	Activity Scale[c]	Est. Activity Scale
1	0.5	0.5	–1.0	9.28	+++	+++
2	2	2.6	1.3	8.56	+++	+++
3	2.4	3.9	1.6	8.38	+++	+++
4	4.3	19	4.4	7.70	+++	+++
5	9	13	1.5	7.85	+++	+++
6	9.4	72	7.6	7.12	+++	+++
7	18	81	4.5	7.07	+++	+++
8	30	73	2.4	7.11	+++	+++
9	60	85	1.4	7.05	+++	+++
10	70	87	1.2	7.04	+++	+++
11	70	85	1.2	7.05	+++	+++
12	84	69	–1.2	7.14	+++	+++
13	180	140	–1.3	6.84	++	++
14	280	68	–4.1	7.14	++	+++
15	310	79	–3.9	7.08	++	+++
16	330	70	–4.6	7.13	++	+++
17	400	78	–5.2	7.08	++	+++
18	460	380	–1.2	6.39	++	++
19	500	910	1.8	6.01	++	++
20	770	250	–3.1	6.58	++	++
21	2500	13000	5.3	4.86	+	+
22	3200	1200	–2.6	5.89	+	+
23	16000	23000	1.5	4.60	+	+
24	50000	15000	–3.4	4.80	+	+
25	100000	59000	–1.7	4.21	+	+

[a]+ indicates that the estimated IC50 is higher than the experimental IC50; – indicates that the estimated IC50 is lower than the experimental IC50. [b]Fit value indicates how well the features in the pharmacophore overlap the chemical features in the molecule. [c]P56 LCK activity scale: +++, IC50 ≤ 100 nM (high active); ++, 100 nM < IC50 < 1000 nM (moderately active); +, ≥ 1000 nM (inactive)

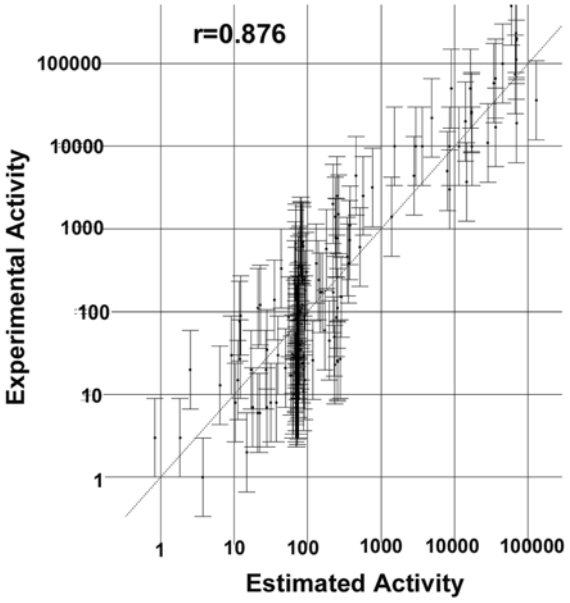

Figure 3. Correlation between experimental and Hypo1 estimated activities.

the runs are listed in Table 3. The data of cross validation clearly indicates that all values generated after randomization, produced hypotheses with no predictive value near to or similar to the original one. Out of the 19 runs, only one had a correlation close to 0.80, but the RMSD was high and total cost was close to the null cost, which is not desirable for an acceptable hypothesis. This cross validation also provided strong confidence on the initial pharmacophore model, Hypo1 and thus has been concluded as the best ranked pharmacophore hypothesis.

The best ranked validated pharmacophore model, Hypo1 was used as a search query to retrieve molecules from chemical database. Activities were estimated by Hypo1 which retrieved 9% hits from NCI chemical database. Among those, we have considered hits that showed less than 5 nM of the estimated activity (657 hits) for further evaluation. The mapping of Hypo1 on two hits found in NCI database search named as NCI0310811 and NCI0303873 are represented in Figure 2e and Figure 2f. The estimated activities for these two compounds are 0.42 nM and 0.55 nM with fit values of 9.352 and 9.238 respectively. Training set compounds as well as 657 new hits were docked into P56 LCK ATP binding site using *GOLD* docking software. As the interacting ability of a compound depends on the fitness score (overall value representing van der Waals and electrostatic interactions), the greater the *GOLD* fitness score the better the binding ability. Hence fitness score of 50 was taken as cut-off for considering them for next step of filtration. Thus 203 hits were obtained which had with fitness score greater than 50.

We have analyzed drug-like properties based on Lipinski's rule-of-five for these 203 compounds by using *Molinspiration* online database. According to the rule-of-five model, compounds are considered likely to be well absorbed when they possess LogP less than 5, molecular weight less than

Table 3. Results from cross-validation using *Cat-Scramble* in *CATALYST* [a]

Trail No.	Total cost	Fixed cost	RMSD	Correlation (r)
	Results for unscrambled			
	112.675	100.775	0.933	0.941
	Results for scrambled			
1	165.611	95.237	2.372	0.507
2	177.196	95.728	2.540	0.388
3	134.007	97.571	1.642	0.806
4	151.695	96.160	2.090	0.652
5	152.017	98.375	2.068	0.660
6	156.822	97.176	2.138	0.637
7	173.173	93.145	2.530	0.395
8	159.561	96.547	2.243	0.580
9	156.129	93.180	2.196	0.613
10	160.064	96.436	2.255	0.574
11	137.457	97.565	1.735	0.779
12	147.727	95.461	2.011	0.686
13	162.605	95.471	2.280	0.570
14	175.504	96.851	2.497	0.423
15	164.921	94.860	2.364	0.513
16	138.050	98.651	1.736	0.778
17	157.091	94.116	2.228	0.590
18	147.440	97.934	1.987	0.692
19	159.843	97.512	2.232	0.585

[a]Null cost = 178.9

500, number of H-bond donors less than 5, and number H-bond acceptors less than 10. We have also confined the number of rotatable bonds not to exceed 10. Reduced molecular flexibility, as measured by the number of rotatable bonds and total H-bond count (sum of donors and acceptors) are found to be important predictors of good oral bioavailability. Drug-like properties of the each compound can be calculated based upon the structure and therefore SMILES (Simplified Molecular Input Line Entry System) were generated for the 203 hits using *ISIS/ConSystant* program and were used as an input into *Molinspiration* database which gives information about miLogP, molecular weight, number of H-bond donors, acceptors and rotatable bonds. Finally 68 compounds have satisfied all the physiological properties to be an ideal lead molecule. Thus these compounds have satisfied all the above three filtering methods of good predictive activity, good docking scores and also drug like properties.

We have analyzed docking results of highly active compound in training set, two test set compounds and two highly estimated active compounds from the database search. Three H-bond interactions, two with Met319 and other with Thr316 formed by all the above mentioned compounds as shown in Figure 4 are considered to be crucial for inhibitory activity. The two hits NCI0310811 and NCI0303873 are adenosine derivatives. The amino group of amide in training and test set compounds act as an H-bond donor to the oxygen of Thr316 as shown in Figure 4. Pharmacophore mapping as shown in Figure 2 indicates that the amino group of amide acts as HBD. In the case of adenosine derivatives the 5'-OH

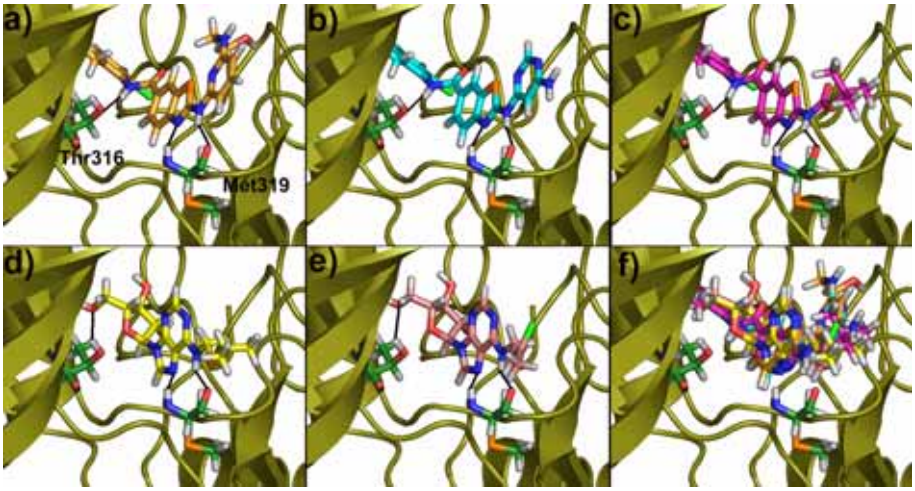

Figure 4. Molecular docking of training set compound 1 (a), test set T24 (b), T35 (c) New compound NCI0310811 (d) and NCI0303873 (e) aligned ligands (f) at P56 LCK ATP binding site.

of ribose ring is acting as H-bond acceptor from the hydroxyl group of Thr316 while the amino group is acting as H-bond donor to the Met319 carbonyl group. Pharmacophore mapping also presented similar feature mapping for these adenosine derivatives. Adenosine derivatives were substrate analogues as ATP is the substrate at the P56 LCK ATP binding site. Our training set compounds had no adenosine analogues and it is surprising to know that the pharmacophore model was able to retrieve adenosine derivatives based on the validated pharmacophore model features.

Conclusions

For P56 LCK inhibitor dentification, a reliable pharmacophore model with four crucial features HBA, HBD, HY-ALI and RA which could identify and differentiate active from inactive compounds was developed. The model was validated with 178 test set compounds and also by *Cat-Scramble* method. In drug design process identifying compounds with good inhibitory activity is the crucial and basic step. The validated pharmacophore was used to retrieve new compounds with estimated inhibitory activity in nanomolar range from NCI database. New compounds were refined and identified by the following two additional filtering methods: molecular docking and drug-like property check. Molecular docking score determines whether the pharmacophore retrieved new compounds bind to the active site residues of the target similar to the experimentally proved inhibitors. This molecular docking filtering method plays crucial role as compounds with good dock scores as well as interactions with crucial amino acids were considered as cutoff to eliminate compounds which lacked such properties. Drug-like properties like molecular weight, LogP, number of H-bond donors, H-bond acceptors, and molecule flexibility measured as rotatable bonds were evaluated to ensure proper physiological properties. The second filtering method helps us to eliminate compounds which though have good estimated inhibitory and binding properties lack the essential property to be a candidate molecule. We have identified 68 new compounds with better estimated activity, good calculated binding properties and also favorable drug-like properties based on Hypo1 from NCI database consisting of 238,819 compounds. Among 68 compounds interestingly few adenosine derivatives were found. This can be possible as the pharmacophore model features complement the P56 LCK ATP binding site. Thus, the validity of the pharmacophore model could be substantiated as it could retrieve adenosine derivatives although no such compounds were included in the training set. Therefore our pharmacophore hypothesis is reliable and is able to predict new compounds with good estimated activity from any chemical database. Thus, among the final 68 compounds few may have good *in vitro* P56 LCK inhibitory activity.

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