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Structure based de novo design of novel glycogen synthase kinase 3 inhibitors

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Abstract—Glycogen synthase kinase 3 (GSK-3) is a serine/threonine kinase that has captured great attention in drug discovery projects. Structure based design has been successfully carried out to find a novel class of GSK-3 inhibitors using the Ludi de novo ligand design program. A total of 15 potential leads are suggested from the study. The structures have been validated through detailed analysis of the Ludi score values and by molecular docking experiment using FlexX. The hits have been further verified through: (1) visual examination of how well the hits dock into the GSK-3β binding site; (2) comparative analysis of their FlexX, G_Score, PMF_Score, ChemScore, and D_scores values; (3) a comparative investigation of the docking scores of the hits with those of the reported inhibitors after calibration of the docking procedure with 17 previously reported inhibitors; (4) determination of the binding mode of the hits and comparison with that of the so far known inhibitors. Hits retaining interactions with the common amino acids of GSK-3β binding site were taken to represent potential leads. Structurally the hits designed are mainly flat nitrogen heterocycles. These hits are expected to be important additions to the search of GSK-3 inhibitors and may provide invaluable insights to further understand the structural basis of catalysis and inhibition of this kinase.

1. Introduction

Protein phosphorylation and dephosphorylation are important processes in the control of protein functions. Biological phosphorylation mostly occurs on hydroxyl containing amino acids such as serine, threonine, and tyrosine, and is catalyzed by protein kinases whose number exceeds 500 in the human genome. The importance of protein phosphorylation as a main regulatory mechanism used by cells to regulate enzymes and other proteins and the association of many maladies with its aberrations[1] make kinases to be important targets and the hunt for kinase inhibitors has attracted a great attention in drug discovery in recent years.[2–7] Glycogen synthase kinase-3 (GSK-3) was originally identified and studied for its function in the regulation of glycogen synthase,[8–10] the rate determining enzyme in glycogen biosynthesis.[11] It is a cytosolic serine/threonine kinase which exists in two isoforms (α and β) in mammals. These isoforms share high homology (>95%) at the catalytic domain and are expressed ubiquitously in cellular systems and generally have similar biochemical properties.[12] GSK-3 has multiple substrates[13,14] and plays a critical role in glucose homeostasis,[15] CNS function, and cancer,[13] circadian rhythm, cell death, cell survival, and others. Judging from the importance of phosphorylation in cellular and physiological events and the diverse substrates these kinases have, GSK-3 inhibitors have got a wide spectrum of therapeutic potential among which diabetes, neurodegenerative diseases,[16] bipolar disorders,[17] stroke, cancer, and chronic inflammatory diseases[18] are the major ones.

Figure 1 shows some classes of GSK-3 inhibitors. There are a number of reported GSK-3 inhibitors including hymenialdisines,[19] paullones,[20] indirubins,[21] and maleimide (bisarylmaleimides,[22] anilinomaleimides,[23] bisindolylmaleimides,[24] azaindolyl maleimides[25]) derivatives. Despite the chemical diversity of the so far reported inhibitors and the huge potential of GSK-3 inhibition, currently there is no small molecular drug

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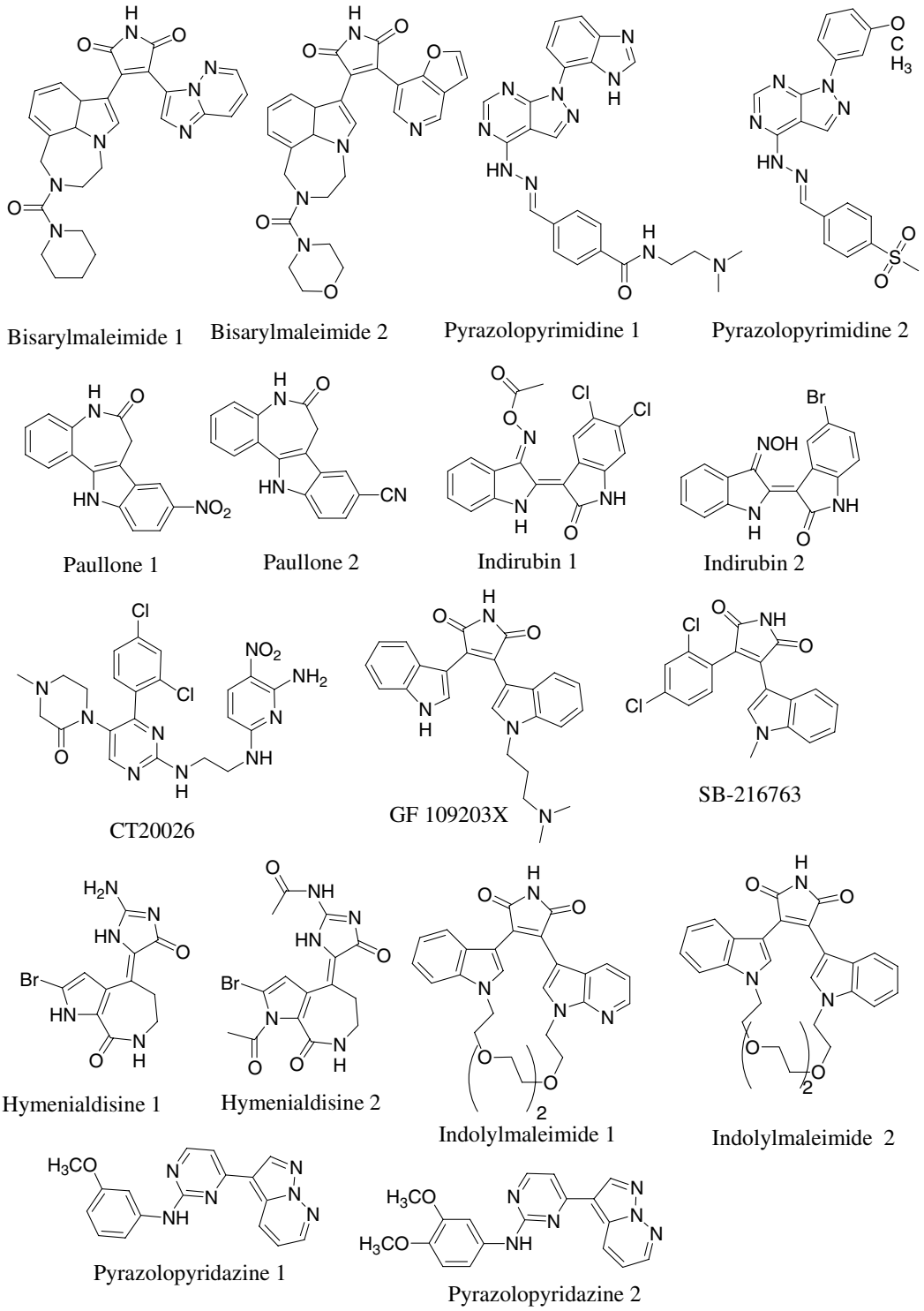

Figure 1. Structures of reference compounds used for calibration of the docking methodology.

that acts via GSK-3. The major reason hampering all efforts is the non-selective nature of the so far reported inhibitors, that is, in addition to binding GSK-3, the so far reported inhibitors also bind other kinases too. Since this non-selectivity is accompanied by unwanted drug effects, agents that bind selectively to GSK-3 isoforms are expected to have a better therapeutic profile. Most of the so far known inhibitors are discovered through high throughput random screening experiments. To alleviate the non-selectivity issue ligand design techniques which explicitly take into account the information from the three-dimensional structure of the biomolecular target are worthwhile. Such information could be usefully employed to bias the nature of the association of the ligand with its target thereby helping to attain an acceptable level of selectivity.

Structure based de novo design[26,27] is best suited in this case. The methodology first derives information about the putative binding site of the target of interest. The information includes the steric, hydrophobic, electrostatic, van der waals, hydrogen bonding, and other characteristics of the binding groove which are important in interacting with a potential drug. Within the limits of these constraints, a structure is then generated by applying the principle of complementarity. Structures are then scored, ranked, and further evaluated and verified. There are several successful applications of structure based de novo design efforts. Matsunaga et al.[28] employed structure based approach to design novel and potent non-steroidal $C_{17,20}$-lyase inhibitors with nanomolar activities. Terasaka et al.[29] have succeeded in the de novo design of a non-nucleoside type of human adenosine deaminase (ADA) inhibitors using the three-dimensional structure of ADA complexed with known nucleoside inhibitors. Their work reveals the discovery of 1-(1-hydroxy-4-phenyl-2-butyl) imidazole-4-carboxamide (FR221647: $K_i = 5.9$ mM to ADA) as a novel inhibitor with moderate activity and good pharmacokinetic properties compared to the known inhibitors. Structure based design tools such as Ludi[30,31] which heavily employ the ligand–receptor complementarity principle are important in attaining an acceptable level of selectivity. Ludi has been successfully applied by Grembecka et al.[32] to design and predict the activity of leucine aminopeptidase inhibitors. Zhu and co-workers have applied the Ludi program to rationally design tRNA-guanine transglycosylase inhibitors.[33] Ludi works in three steps. First, it calculates interaction sites within the protein's active site; second, it searches libraries for fragments and fits them onto the interaction sites; third, it proposes an alignment or linking for the fragments. The Ludi program recognizes hydrogen bonding and hydrophobic interactions between a ligand and a receptor. This is particularly interesting as these interactions are the ones that contribute predominantly for inhibitor binding with GSK-3β.[34] These attributes make Ludi an important tool in de novo design of GSK-3 inhibitors.

2. Results and discussion

With the 3D structure of drug targets on the increase, it seems appropriate to apply a methodology that is suitable in the initial phase of drug discovery. Ludi is based on fragmental approach to de novo ligand design. It is purely geometric and as such it avoids costly potential energy calculations. In the present study, the de novo mode of the program yielded 11 hits. The several preset parameters employed during hit generation are meant to remove unpromising structures. So, the 11 hits obtained are what Ludi thinks have satisfied the minimum of all defined parameters out of thousands of possibilities. Ludi employs an empirical scoring function to compute the free energy of binding and hence to prioritize the hits obtained. Whenever a fragment can fit in multiple orientations only the highest scoring fit is retained. The highest total Ludi score obtained was 304. Since the scoring function used in Ludi gives a measure of the free energy of binding, hits with Ludi score of greater than 200 were chosen for a further investigation. This operation resulted in five structures out of a total of 11 hits. The structures of these top-scoring five hits obtained from the initial de novo run are shown in Figure 2. Analysis of the different components of the score shown in Table 1 reveals that the hit with the highest Ludi score has also the highest hydrogen bonding score (261) and it has the second highest contact score (63). However, the lipophilic score (139) of this hit was the lowest of the top five hits. Nevertheless, as there is a big difference in the contribution of hydrogen bonding to the total score and because of the importance of hydrogen bonding to interact with GSK-3β[34,35] this top scoring Ludi hit was retained for further study. In order to select one molecule from the remaining, FlexX based docking was used to assist with the evaluations. In addition to its

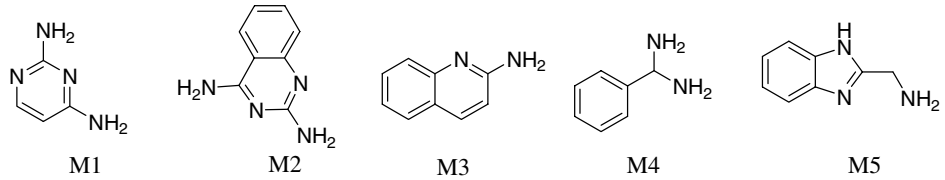

Figure 2. Structures of the hits from the standard de novo run.

Table 1. Ludi and other docking scores of ligands obtained using de novo mode of Ludi

Hit No.	Total LUDI score	HB score	Lip score	Rot score	Contact score	RMSD	FlexX	G_score	PMF score	D_score	CHEM Score	CScore
m1	304	261	139	0	63	0.64	−14.46	−47.39	−37.24	−32.13	−17.43	4
m2	221	87	230	0	55	0.75	−25.30	−56.52	−44.95	−45.31	−24.78	4
m3	215	27	284	0	67	0.65	−15.83	−69.73	−26.40	−51.68	−21.96	4
m4	205	79	222	0	57	0.40	−15.97	−60.90	−9.80	−43.86	−17.81	2
m5	204	89	236	−25	52	0.52	−19.00	−90.00	−30.88	−44.26	−17.28	3

Table 2. Docking scores of ligands obtained using AutoLudi

Compound No.	LUDI score	Docking scores					
		FlexX	G_score	PMF score	D_score	CHEMScore	CScore
m11	1008	−32.82	−257.58	−78.36	−145.26	−42.89	5
m12	834	−32.18	−244.18	−71.14	−137.28	−37.88	4
m13	851	−32.0	−260.02	−59.29	−144.14	−41.39	5
m14	875	−31.52	−240.02	−70.27	−133.59	−40.09	3
m15	830	−29.75	−47.50	−48.36	−137.63	−30.41	3
m16	1020	−29.65	−143.01	−15.57	−106.15	−39.25	2
m17	883	−29.34	−249.85	−61.09	−135.48	−38.60	5
m18	857	−28.99	−262.67	−49.43	−137.97	−35.39	3
m19	826	−27.17	−253.70	−52.85	−137.80	−39.54	5
m110	826	−26.27	−257.89	−55.42	−135.60	−37.37	5
m211	884	−30.92	−262.42	−38.50	−168.98	−33.16	4
m212	826	−28.43	−179.27	−46.96	−100.20	−31.62	4
m213	910	−28.16	−159.81	−2.82	−135.22	−32.62	3
m214	852	−26.34	−145.94	−56.54	−111.16	−29.82	4
m215	780	−26.08	−143.27	−40.80	−126.91	−25.64	3

Note: Compound number with a prime indicates hits obtained using M4 as a scaffold, whereas the rest are obtained using M3 in the AutoLudi run.

total Ludi score, the scores of the three scoring functions (i.e., FlexX (−25.3), PMF (−44.95), and ChemScores (−24.78)) were found to be better for the second high scoring Ludi hit. From this initial run, the two hits with high Ludi scores were found worthy of further consideration and were taken for the AutoLudi program. The Ludi and docking scores of all the five hits are shown in Table 1. AutoLudi uses a starting scaffold onto which other fragments are linked in an automated fashion. All the hydrogen atoms on the hits were determined as potential link sites for the AutoLudi run. The AutoLudi program was set to display only the top scoring 50 hits. Use of the top Ludi hit from the standard de novo run yielded 45 structures. The Ludi score does improve drastically as is evident from Table 2. The maximum Ludi score obtained was 1020 with the next highest being 1008. In order to evaluate the binding affinity with more scoring functions, the molecules were constructed in SYBYL and were docked into the GSK-3β binding site using the FlexX docking software. The CScore module was also used apart from FlexX. This is very important as the different approaches use different assumptions and approximations and hence have their own inherent limitations. By use of different scoring functions, it is hoped that the limitations of one function may be overcome and hence much more fair comparisons can be made in rank-ordering the hits and to compare their predicted binding affinities with existing GSK-3 inhibitors. In order to be assured of the docking experiment we needed to assess how the FlexX and other docking scores used predict the affinities of known inhibitors which have an experimentally determined IC_{50} values.

Table 3. IC_{50} and docking scores of reference compounds

S. No	Name	IC_{50} (Ref.)	Docking scores					
			FlexX	G_Score	PMF score	D_Score	ChemScore	CScore
1	Bisarylmaleimide 1	0.7 nM[22]	−35.13	−256.53	−27.62	−138.91	−38.24	3
2	Bisarylmaleimide 2	0.8 nM[22]	−30.71	−282.25	−30.56	−142.95	−36.67	2
3	Indirubin 1	4 nM[21]	−29.76	−174.59	−45.06	−105.89	−31.64	5
4	Indirubin 2	5 nM[21]	−29.84	−175.06	−47.89	−99.10	−33.57	5
5	Paullone 1	4 nM[20]	−26.91	−126.69	−39.12	−84.14	−35.62	4
6	Paullone 2	10 nM[20]	−21.32	−150.40	−27.31	−83.56	−31.62	4
7	Hymenialdisine 1	10 nM[19]	−23.44	−141.48	−41.45	−79.70	−23.73	4
8	Hymenialdisine 2	130 nM[19]	−21.39	108.56	−26.31	−43.18	−20.52	2
9	Pyrazolopyridazine 1	10 nM[48a]	−21.54	−60.29	−41.20	−58.87	−30.62	5
10	Pyrazolopyridazine 2	12 nM[48a]	−17.14	−58.84	−43.75	−66.44	−27.53	5
11	Indolylylmaleimide 1	17 nM[25]	−14.87	−151.31	−38.67	−68.03	−24.16	5
12	Indolylylmaleimide 2	22 nM[25]	−14.87	−149.98	−38.09	−67.59	−24.22	5
13	Pyrazolopyrimidine 1	8.8[49b]	−27.84	−84.64	−35.35	−120.26	−29.94	4
14	Pyrazolopyrimidine 2	8.6[49b]	−23.10	−114.36	−49.55	−123.94	−30.37	5
15	SB-216763	34 nM[50]	−23.93	−187.76	−34.41	−202.87	−33.35	5
16	CT2006	4 nM[51]	−28.53	−101.69	−62.30	−96.41	−28.60	4
17	GF109203	190 nM[52]	−29.39	−253.72	−42.55	−316.77	−35.61	4

[a] Stands for which the K_i instead of IC_{50} is reported.

[b] Refers to the pIC_{50}: numbers after the compound name indicate for the most active molecule(1) and for the second most active molecules(2) in the series reported. Ref., literature reference of the bioactivity.

For this purpose, 10 different known ligands representing the most active molecules from their respective classes were taken from the available literature and were docked into the same GSK-3β binding site the hits were docked using the same methodology. The docking scores of the reference molecules are shown in Table 3. As this table shows, the FlexX score varies from −14.37 to −35.13 for the references, the average being −24.5. The FlexX score of the initial five Ludi hits varied from −14.46 to −25.30 which is still within the range of the scores of the known inhibitors. As FlexX tries to predict the binding affinity, the values for the hits found suggest some potential applicability. As the known inhibitors are reported by different groups and in different laboratories using not necessarily identical protocols direct comparison of IC_{50} with docking scores is obviously difficult. Nevertheless, a hit with FlexX score comparable to the reported inhibitors should ideally present a potential lead. It was suspected that everything that contributes to the differences in docking scores should be inherent to the nature of ligand structure and its affinity for the kinase. In order to evaluate the scores

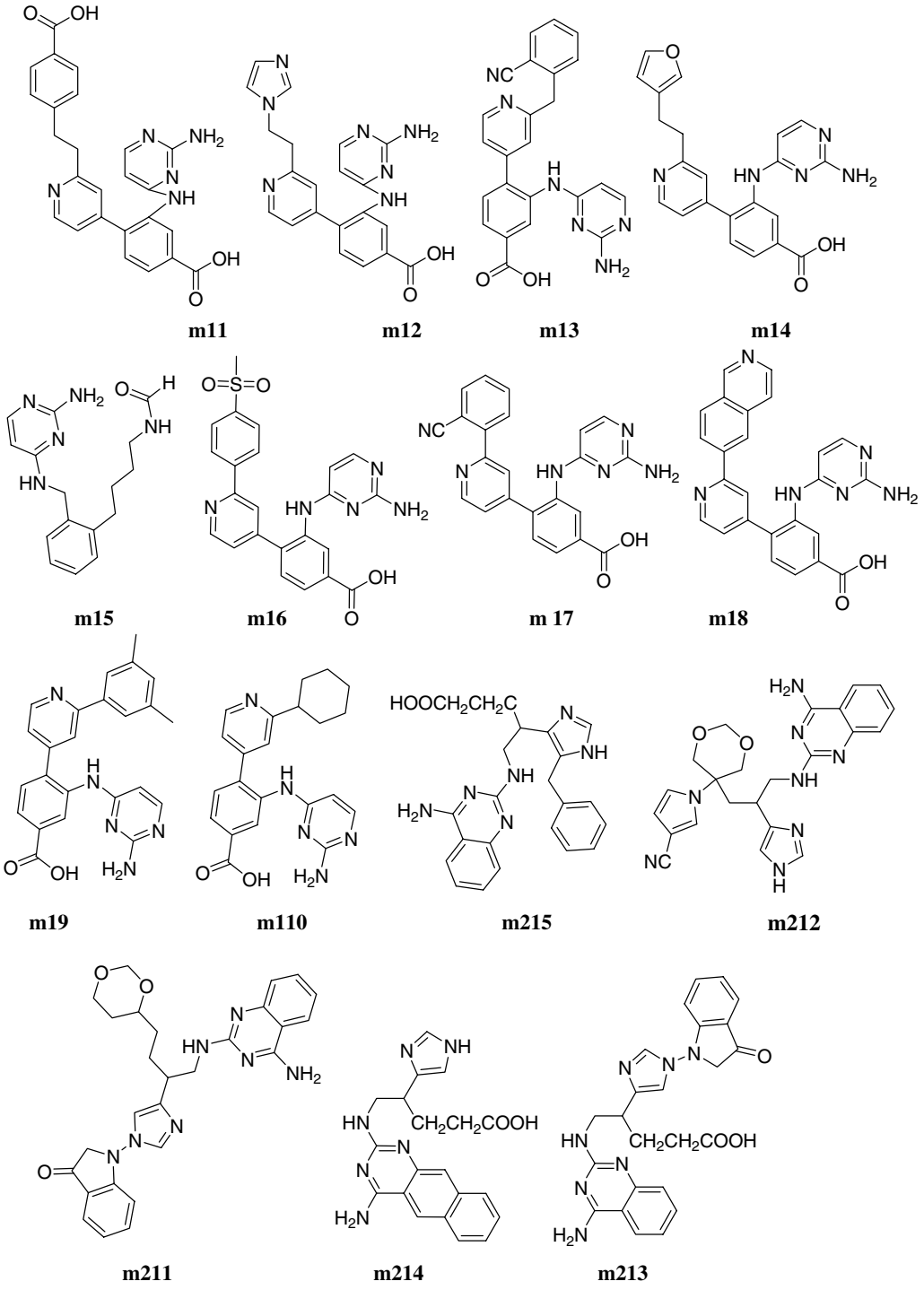

Figure 3. Chemical Structures of Hits from AutoLudi run.

the second most active molecules were taken for docking in order to see how exactly scores correlate with the inhibitory potencies of the inhibitors. Seven of the molecules were reported in a series and the next most active molecules were taken for the docking study. The results are displayed together with most active 10 molecules in Table 3. Of the docking scores obtained, the result shows how well FlexX score parallels experimental IC_{50} quite accurately. In the molecules taken, FlexX score appears in most cases to predict the correct order of each series and thus provide some confidence in the relative order of molecules in terms of their affinities. And hence FlexX score was used as a basis to verify compounds that would be expected to bind with a higher affinity. Hits with a FlexX score better than −26.00 (above the average for the so far reported inhibitors) were taken to represent novel potential leads. This returned 10 out of the initial 45 Ludi hits obtained using the best scoring scaffold from the initial standard de novo run. Next the second top Ludi scoring fragment was employed to carry out the AutoLudi run. This study provided 29 hits in total. These too were docked into GSK-3β binding site using FlexX and the other scoring functions were also requested in a similar way. The highest Ludi score obtained was 910. Apart from the FlexX score, the scores from the other scoring functions were also used during the structure verification. In most of the cases, the PMF, Chem, G_, and D_scores do show a degree of correlation with the experimental IC_{50} and hence these too were used in the verification. The PMF, Chem, D_ and G_scores are just estimates of binding free energy as is the FlexX and other molecular docking scores.As these are measures of the free energy of binding, the more negative (lower) it is, the better will be the binding. Positive value means there is unfavorable binding or association and hence it indicates unpromising ligand. Seven molecules were found to have a FlexX score better than −26.00. However, examination of the other scoring functions revealed that two of these were having positive G_and D_scores. As this is an unlikely scenario these were removed and only 5 potential leads were obtained using the m2 scaffold. As can be seen the CScore values for the hits are mostly better than the reference molecules. Some of the hits do have protonated ring nitrogen which is also a common structural element as is evident from the structures of the reference molecules in Figure 1. The final hits remained were then visually observed how they dock into the GSK-3β binding site. Lastly, the retention of interactions with the important amino acid residues of the GSK-3β binding site is used as a final check. Inhibitors of GSK-3 that are reported thus far interact with common residues such as Val135, Arg141, Asp133, Gln185, and Asn186.[37] The hits were found to interact with these residues thereby illustrating their mechanistic homogeneity with the reported inhibitors. These 15

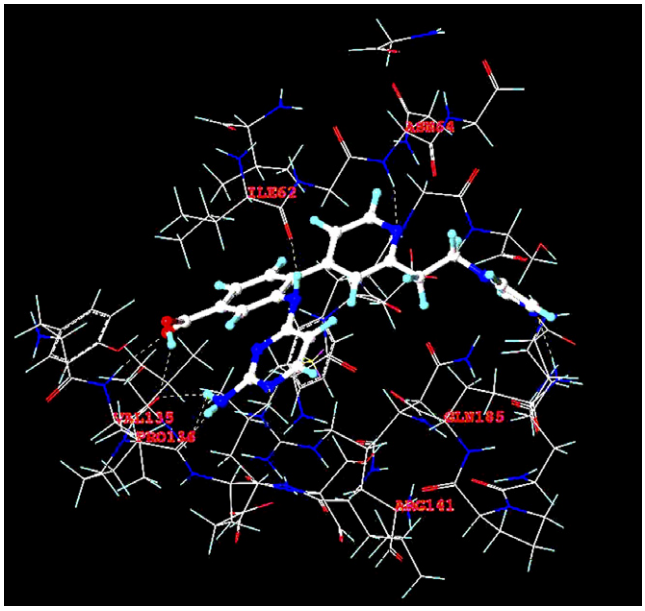

Figure 5. Interactions of m12 with GSK-3β active site.

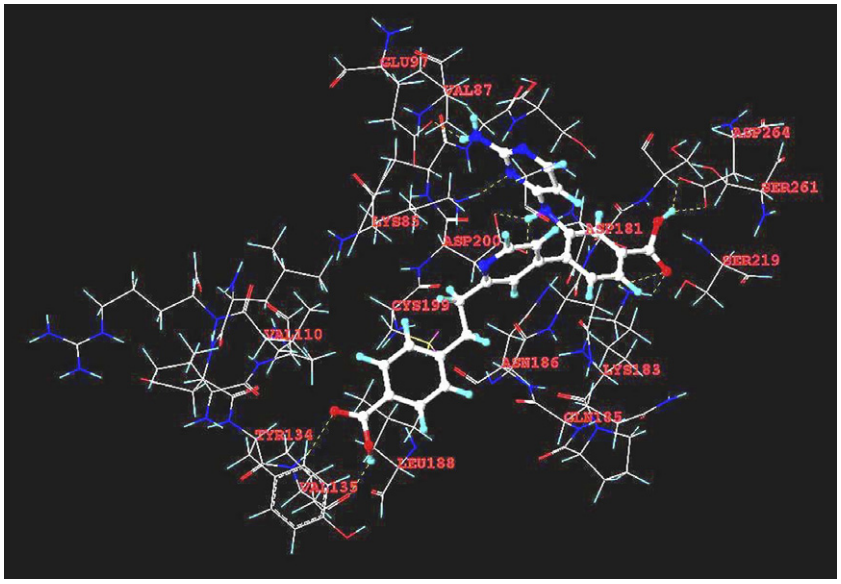

Figure 4. Interactions of m11 with GSK-3β active site.

molecules are expected to be new potential leads. Considering the simplicity of their structure, the hits are expected to be synthetically tractable. The structures of these hits are shown in Figure 3. The binding modes of the FlexX docked structures were compared with those of the experimentally known inhibitors not with Ludi docked structure for there is no previously reported Ludi pose to make the comparison. The interaction of the two highest FlexX scoring hits is displayed in Figures 4 and 5.

2.1. Interactions of ligand m11 with GSK-3β active site

The interaction of m11 is shown in Figure 4. The display shows that the COOH on the phenyl ethylene of the hit is making hydrogen bonding interactions with NH of Val 135. Apart from this the other carboxyl group on the anilinophenyl ring is also making favorable hydrogen bonding interactions with the Asp264 and Lys183. The amino (NH_2) of the hit is shown to be involved in hydrogen bonding interactions with the carbonyl of the Glu97, while the NH that bridges the pyrimidine and the phenyl ring is involved in making hydrogen bonding interactions with Asp200. The NH_2 of Lys85 is also noticed to make hydrogen bonding interactions with the nitrogen atom on the pyrimidine ring. A hydrophobic interaction appears to occur between Leu188 and Val110 of the GSK-3β binding site with the phenyl ring of the hit.

2.2. Interactions of m12 with GSK-3β active site

The docked interaction for hit m12 is shown in Figure 5. The pyrimidine nitrogen of the hit is seen to make hydrogen bonding interactions with the Arg141. The amino of the hit is observed to make hydrogen bonding interactions with the carbonyl of Pro136, while the NH that bridges the pyrimidine and the phenyl ring is involved in making hydrogen bonding interactions with Ile62. Moreover, the carbonyl of the COOH moiety on the anilino phenyl ring is also seen to make hydrogen bonding interactions with the NH of Val 135. Another hydrogen bond is seen between the pyridine nitrogen of the hit and the NH of Asn64. The NH_2 of Gln185 is involved in hydrogen bond formation with the imidazole nitrogen. Finally, a potential hydrophobic interaction does appear to exist between Ile62, Leu188, Leu132 and with the phenyl and the pyrimidine rings of the hit. These amino acids of the GSK-3β active site are considered to be important in interacting with known inhibitors from earlier study.[37] The two hits were able to reproduce these important interactions thereby illustrating their potential utility as inhibitors.

3. Conclusions

Structure based de novo ligand design has been successfully carried out to design novel potential leads. A total of 15 validated potential leads are suggested from the study. Structure verification has been further carried out by molecular docking using FlexX. As a preliminary filter visual examination on how well the hits fit into the GSK-3β binding site has been made. Following this a comparative analysis of their FlexX, G_Score, PMF_Score, ChemScore, and D_scores values coupled with calibration of the docking experiment using 17 previously reported inhibitors has been effected in order to better rank-order the hits obtained and to have confidence on the docking methodology. A comparative investigation of the docking score of the hits with that of the reported inhibitors demonstrates the potential these hits have as GSK-3 inhibitors. Finally, the binding modes of the hits have been determined and those that retain interactions with the common amino acids of the GSK-3 binding site were taken to represent potential leads. Structurally, the hits are mainly flat hydrophobic nitrogen heterocycles.

4. Methodology

The co-ordinates of GSK-3β were obtained from the refined X-ray structure of human GSK-3β in complex with AMP-PNP which is available from the Brookhaven Protein Data Bank (accession code:1PYX.pdb). The hydrogen atoms were added using Cerius^2 program.[36] There was no minimization after additions of hydrogen atoms. Water molecules except those that were found to be important in aiding interaction between the receptor and previously reported inhibitors[37] were removed. The inhibitor AMP-PNP which was in complex with the enzyme was used to define the active site. The active site was defined within 8 Å from the center of the AMP-PNP inhibitor. The maximum RMSD was set to 0.5 Å. The RMSD refers to the allowed tolerance of matching appropriate fragment atoms to the required interaction points. The program Ludi was applied to design GSK-3β inhibitors on Silicon Graphics Fuel workstation. Ludi is based on the fragment approach to lead discovery. Ludi identifies four different types of interaction site: lipophilic aliphatic, lipophilic aromatic, hydrogen bond donor, and hydrogen bond acceptor. It suggests how suitable small fragments can be positioned into clefts of protein structure in such a way that hydrogen bonds can be formed with the enzyme and hydrophobic pockets are filled with hydrophobic groups. This positioning is the strength of Ludi because it immediately provides with ideas about how putative binding sites on the protein might be saturated by fragments and how those fragments might be linked together. First the de novo mode of the program was used to generate novel scaffolds and hence the standard de novo library of Ludi was requested initially. A systematic search of Ludi's fragment library containing about 1000 structural fragments was performed. The Energy_Estimate scoring function was used that estimates the change in free energy upon binding of the fragment to the receptor. Each fragment is evaluated as a function of the potential number of hydrogen bonding, hydrophobic and ionic contacts it can make and an estimate is made of the penalty due to freezing the internal degrees of freedom of the ligand. Unless otherwise stated standard Ludi parameters were applied. Next the two top scoring hits from the standard de novo run were taken for the

AutoLudi operation. AutoLudi uses the existing Ludi program for de novo drug design. Given a scaffold positioned in a protein site, AutoLudi uses Ludi (i.e., the inhibitor program in link mode) to identify fragments that may be covalently fused to the scaffold and produces a collection of molecules with high Ludi scores. The nature of fragment selection and construction of new molecules depends on the mode that AutoLudi is running. AutoLudi offers three modes of generating hits; Evolutionary mode, Quick mode, and CombiMode. The evolutionary and quick modes are used in a rational design experiments, while the CombiMode is employed to generate combinatorial libraries. In the present study, the evolutionary mode was applied as it is more suitable once we have the basic scaffold obtained from the initial standard de novo run. Important AutoLudi parameters that were employed include: maximum number of generations was set to 3; maximum family size was also set to 3; maximum population size was set to 50. In order to have a standard geometry, the energy minimize option was also requested. Then the Lipinski's rule of five[38,39] was applied so as to avoid generating unpromising structures; maximum molecular weight $\leqslant$500, number of hydrogen bon donors $\leqslant$5, number of hydrogen bond acceptors $\leqslant$10. The number of rotatable bonds was also set to $\leqslant$8. Unless otherwise stated, the other AutoLudi parameters were kept at their default.

4.1. Molecular docking studies

In order to have a broader picture of the binding mode and binding affinity, molecular docking[40,41] was carried out on the hits obtained. The FlexX[42] program interfaced with SYBYL6.9[43] was used. FlexX employs a fast algorithm for flexible docking of small ligands into a fixed protein binding site using an incremental construction process. Standard parameters of the FlexX program as implemented in SYBYL6.9 were used during docking. To further evaluate the docking experiment, the G_score,[44] PMF_Score,[45] D_score,[46] and ChemScore[47] values were estimated using the Cscore module of SYBYL. Since Cscore is a consensus scoring function, the different scoring functions in it provide multiple approaches to better evaluate ligand–receptor interactions. The higher CScore value is associated with better promising hits. Also to compare the scores of the Ludi hits with the existing inhibitors, structurally diverse 17 known GSK-3 inhibitors were docked into the same GSK-3β binding site using the FlexX methodology. This was done to see how the docking methodology accurately predicts known binding affinities. The binding affinities have been predicted by five scoring functions and compared to the experimentally known inhibitory concentrations.

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