ABSTRACT

Several virtual screening techniques are currently available to rapidly screen compounds from virtual compound databases. In virtual screening, computational models are used to predict the biological activity of compounds. The computational models can be generated and validated utilizing either the 3D structure of the target or a set of active analogues specific to the target. Computational models can also be built combining information from structure of the drug target and a set of active analogues specific to the target.

Utilizing these technologies to discover small-molecule ZAP70 and HSP90 inhibitors, we employed consensus models generated by combining both structure-based and active analogue-based methods. In this study, we present the pharmacophore modeling, the docking studies, and finally the use of the consensus models to discover a set of novel ZAP70 and HSP90 inhibitors with biological activity in HSP90-overexpressing SKBR3 cells.

I. Design of ZAP70 inhibitors

Zeta-chain-associated protein kinase 70 (ZAP70 kinase), a 70 kDa member of the Syk family kinase associated with the zeta-subunit of the T-cell receptor, is primarily expressed in T and NK cells. It plays an
important role in the initiation of normal TCR signaling, crucial for T-cell activation and development. Human severe combined immunodeficiency patients completely lack the expression of ZAP70 indicating that ZAP70 is an important target for immunesuppression which is immensely useful in autoimmune disorders and transplant rejections.

The three dimensional structure of the catalytic tyrosine kinase domain of ZAP70 has been crystallized recently in complex with Staurosporine at 2.3 Å resolution. Staurosporine is a non-selective, ATP inhibitor of many kinases having IC$_{50}$ value of 55.8 nM against rhZAP-70 tyrosine kinase. There is currently no highly specific, small-molecule inhibitor for ZAP70. In situations where overactive T cells are a substantial component of disease, such as in autoimmune diseases or transplantation, targeting ZAP70 could provide a means to target the T cells while avoiding adverse effects on other cells. Thus, a potent and selective ZAP70 tyrosine kinase inhibitor should modulate T cell function in a more targeted fashion than the less specific immunosuppressive agents currently available, such as cyclosporine A and FK-506.

Strategy as per the given flowchart was followed to identify potential ZAP70 inhibitors.
Compounds collection - 5729 ZAP70 inhibitors from 246 journal articles and patents

Selection of 135 human ZAP70 inhibitors based on the chemical and biological diversity

Analog-based

Selection of training set and Test set

- HipHop – 6, 129

Generation of Series of Pharmacophores using training set

Validation of the pharmacophore models

Selection of the best pharmacophore model

Screening of NCI database consisting of 238,819 molecules

- Retrieved 4094 hits (358 high, 1352 medium and 2384 low-active molecules).
  358 highly-active compounds selected for docking studies.

Structure-based

Docking studies on the protein crystal structure (1U59) using GOLD software

Correlation of the GOLD fitness score with the biological activity (135 molecules, $r = 0.712$).

Generation of good virtual screening model

Top 6 hits selected based on the GOLD fitness score.
GVK BIO Online Structure Activity Relation database (GOSTAR – www.gostardb.com) documents 5729 ZAP70 inhibitors from 246 references (inclusive of Journals and Patents). Out of the available 5729 inhibitors, 135 human ZAP70 inhibitors were selected for modeling studies based on the chemical and biological diversity. These 135 human ZAP70 inhibitors had an activity range (IC<sub>50</sub>) spanning over 5 orders of magnitude i.e. 0.1–50 µM and had similar ZAP70 inhibitory assay.

Qualitative pharmacophore models were generated using a set of highly active molecules. Common feature hypotheses (qualitative models) were produced by comparing a set of conformational models with a number of 3D configurations of chemical features shared among the training set molecules. To confirm essential features prevailing among the ZAP70 inhibitors, 10 common feature hypotheses were generated using the most active molecules. As these models cannot be directly used to predict biological activity of the compounds, quantitative pharmacophore models were generated to predict the biological activities of novel compounds. For the quantitative model generation, 25 training set compounds with structural diversity were taken and classified as highly active (<0.5 mM), moderately active (0.5–5 mM) and inactive (>5 mM). The top ten hypotheses were composed of HBA, HBD, and HRA
features. The values of ten hypotheses such as cost, correlation (r), and root-mean-square deviations (rmsd) were statistically significant. All ten hypotheses were evaluated using a test set of 110 known ZAP70 inhibitors, which were not included in the training set. Predicted activities of the test set were calculated using all ten hypotheses and correlated with experimental activities.

Of the ten hypotheses, Hypo1 showed a better correlation coefficient (0.972) compared to the other nine hypotheses. False positives, false negatives, enrichment, and goodness of hit were calculated to determine robustness of hypotheses. Hypo1 demonstrated excellent prediction of ZAP70 inhibitory activities of the training set compounds.

The best ZAP70 inhibitor model consisted of four-pharmacophore features, (1) one hydrogen bond acceptor, (2) one hydrogen bond donor (3) one hydrophobic aliphatic and (4) one hydrophobic aromatic features. This model picked 4094 hits from a database of 238,819 molecules while 358 molecules were indicated as highly active. Subsequently, docking studies were performed on the hits.

Docking was performed on 135 ZAP70 inhibitors and also on hits retrieved from virtual screening using GOLD. Gold Fitness scores were
compared with observed activity for all molecules which were found to correlate well with the biological activities (135 molecules, $r = 0.712$). The correlation coefficient is $r^2$, which equals to 0.507 and this value is indicative of correlation. The most active molecules were found to have a very high fitness score. A better understanding of the interactions was obtained by viewing the molecules in the active site. The most energetically favorable conformation for molecule 34 in the ZAP70 complex is studied. It forms different binding interactions with the amino acids in the hinge region and other active site amino acids of the ZAP70 kinase. Few novel series of potent leads were suggested based on the interactions energy between ZAP70 and the putative inhibitors which validated not only the virtual screening potential of the model but also identified the possible new chemotypes.
Conclusion: An astute blend of pharmacophore analysis, docking procedures and database search has resulted in predicting putative novel inhibitors of ZAP70 inhibitors which can be used for various immunomodulatory diseases.

Publication:

1. Discovery of potential ZAP-70 kinase inhibitors: Pharmacophore design, database screening and docking studies

   European Journal of Medicinal Chemistry 44 (2009) 4793–4800
   Pubmed ID - 19674816
II. Design of HSP90 Inhibitors

Heat Shock Protein 90 (HSP90), an ATP-dependent molecular chaperone, has emerged as a promising target in the treatment of cancer. HSP90 performs a key function by maintaining the proper folding conformation of various “client proteins”, and inhibition of HSP90 results in misfolded client proteins which are then rapidly degraded by the proteasome. The HSP90 client proteins include many oncogenic signaling proteins such as ZAP70, Her2/ErbB2, Akt, Raf-1, Hif-1a, hormone receptors, survivin, mutant p53, and hTERT etc which are involved in multiple cancers like breast cancer, CLL, GI tumors, multiple myeloma, pancreatic cancer and other solid tumors.

ZAP70 is expressed in patients with aggressive chronic lymphocytic leukemia (CLL). ZAP70+ve CLL cells expressed activated HSP90 with high binding affinity for HSP90 inhibitors, such as 17-allyl-amino-demethoxy-geldanamycin (17-AAG), whereas normal lymphocytes or ZAP70-ve CLL cells expressed nonactivated HSP90. Therapies that specifically target the silencing of ZAP70, and thereby disrupting the enhanced BCR signalling in poor prognosis CLL, would make an appealing drug target for this disease. Current work on HSP90 inhibitors reveals a promising CLL cell specific mechanism for targeting the degradation of ZAP70.
The majority of HSP90 inhibitors developed so far inhibit HSP90 ATPase activity by docking to the N-terminal ATP-binding pocket. This class of HSP90 inhibitors includes natural products Geldanamycin (GA), GA derivatives such as 17-allylamino-17-demethoxygeldanamycin (17-AAG) and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) and Radicicol. Despite the good activity and clinical progression of 17-AAG, which is being studied in various clinical trials, this molecule has several potential limitations including poor solubility, limited bioavailability, hepatotoxicity and extensive metabolism by polymorphic enzymes.

Recently, three synthetic HSP90 inhibitors, with improved pharmacologic profile, have been developed with diverse chemical scaffolds. These inhibitors are being studied for range of cancers in different clinical trials. Currently there are 26 clinical trials, ranging from phase 1 to 3, on 11 HSP90 inhibitors (both 17-AAG derivatives and synthetic inhibitors) for various indications like breast cancer, CLL, GI tumors, multiple myeloma, pancreatic cancer and other solid tumors.

Strategy was to identify the potential HSP90 inhibitors based on the three synthetic HSP90 inhibitors which are proved to have improved pharmacological profile. The following methodology was followed to achieve the same.
Flow chart – HSP90 inhibitors

3 HSP90 inhibitors which are in the clinical trials and have very good pharmacological profile.

Generation of common feature pharmacophore model

Validation of the pharmacophore model with a database of 87 known HSP90 inhibitors and selection of the best pharmacophore

Screening of the In-house GVK BIO database (GOSTAR) with 1 million compounds yielded 5340 compounds

Cluster analysis of the hits resulted in 455 unique cluster representatives

Docking studies of the 455 compounds on the protein crystal structure (2VCI) using GLIDE software yielded 122 compounds

Out of 122 compounds tested against HSP90-overexpressing SKBr3 cell lines, 5 compounds inhibited cell growth with IC$_{50}$ ranging from 2 µM to 32 µM.
Out of the 11 HSP90 inhibitors which are in clinical evaluation, three synthetic small-molecule inhibitors—the purine-scaffold HSP90 inhibitor CNF-2024/BIIB021, the isoxazole derivative VER-52296/NVP-AUY922, and the carbazol-4-one benzamide derivative SNX-5422, were considered in the study as they have improved pharmacologic profile when compared to 17-AAG.

These three synthetic molecules were used to generate common feature pharmacophore models. These models were then validated against a database of 87 known HSP90 inhibitors. The validated pharmacophore model was further used as search query to retrieve molecules with novel structural scaffolds and desired chemical features. These hits were further filtered by docking studies.

Docking studies were performed for 87 known inhibitors with 3D structure of HSP90 having a PDB entry code 2VCI using Glide program. The 87 known inhibitors were docked into the active site of HSP90 and correlation coefficient is calculated between dock score and the pIC\textsubscript{50} using linear regression analysis method. An acceptable correlation coefficient (r) of 0.81 was obtained between experimental pIC\textsubscript{50} and docking energy.
The above correlation proved that the binding modes of the HSP90 inhibitors were reliable and this was further confirmed by the observation that the high active compounds showed better Glide scores than the low active compounds.

Glide generated several feasible bound conformations for each compound and ranked them according to their dock scores. The bound conformation with the most favorable energies was considered as the best binding orientation. Further, the Glide docking program had been applied to screen the hits corresponding to 455 cluster representatives.

Of the 455 compounds, 122 compounds had nice fit into the active site, forming good interactions with the key residues in the protein and showed high dock score.

Based on the docking score, 122 compounds were purchased from the vendor libraries and tested against SKBr3 cell lines, which were shown to over express HSP90.

Of the 122 compounds tested, 5 compounds inhibited cell growth with an IC_{50} value ranging from 2 µM to 32 µM.
<table>
<thead>
<tr>
<th>Compound No</th>
<th>Structure</th>
<th>Cytotoxicity in HSP90 over expressing SKBr3 cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td><img src="image4.png" alt="Structure 4" /></td>
<td>2.05±0.45</td>
</tr>
<tr>
<td>5</td>
<td><img src="image5.png" alt="Structure 5" /></td>
<td>7.01±0.62</td>
</tr>
<tr>
<td>6</td>
<td><img src="image6.png" alt="Structure 6" /></td>
<td>25.27±1.17</td>
</tr>
<tr>
<td>7</td>
<td><img src="image7.png" alt="Structure 7" /></td>
<td>26.97±1.12</td>
</tr>
<tr>
<td>8</td>
<td><img src="image8.png" alt="Structure 8" /></td>
<td>31.27±1.22</td>
</tr>
</tbody>
</table>

Cytotoxicity of pharmacophore hits against HSP90-over expressing SKBr3 cell line.
**Conclusion:** Five structurally-diverse compounds possessing growth inhibitory potency against HSP90-overexpressing SKBr3 cancer cells were identified using pharmacophore, cluster analysis and docking studies. These compounds have IC$_{50}$ ranging from 2 µM to 32 µM. These HSP90 inhibitors can be useful for various therapeutic indications like CLL, breast cancer, multiple myeloma, pancreatic cancer and other solid tumors.

**Publication:**

1. Combined pharmacophore and structure-guided studies to identify diverse HSP90 inhibitors.
   Ramadevi Sanam *, Sunita Tajne, Rambabu Gundla, S. Vadivelan, Pavan Kumar Machiraju, Raveendra Dayam, Lakshmi Narasu, Sarma Jagarlapudi, Nouri Neamati

   Pubmed ID - 20005756