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Combined pharmacophore and structure-guided studies to identify diverse HSP90 inhibitors

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ABSTRACT

Heat Shock Protein 90 (HSP90), an ATP-dependent molecular chaperone, has emerged as a promising target in the treatment of cancer. Inhibition of HSP90 represents a new target of antitumor therapy, since it may influence many specific signaling pathways. Many HSP90 inhibitors bind to the ATP-binding pocket, inhibit chaperone function, resulting in cell death. Recent clinical trials for treatment of cancer have put HSP90's importance into focus and have highlighted the need for full scale research into HSP90 related pathways. Here we report five novel HSP90 inhibitors which were identified by using pharmacophore models and docking studies. We used highly discriminative pharmacophore model as a 3D query to search against database of ~1 M compounds and cluster analysis results yielded 455 compounds which were further subjected for docking. Glide docking studies suggested 122 compounds as in silico hits and these compounds were further selected for the cytotoxicity assay in the HSP90-over expressing SKBr3 cell line. Of the 122 compounds tested, 5 compounds inhibited cell growth with an IC_{50} value less than 50 μM.

1. Introduction

Heat Shock Protein 90 (HSP90), a 90-kDa chaperone, is highly conserved and ubiquitously expressed in all living organisms. It is an attractive molecular target because of its requirement for the stability and function of multiple mutated, chimeric and over-expressed signaling proteins that promote the growth and/or survival of cancer cells. 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 [1]. The HSP90 client proteins include many oncogenic signaling proteins such as ZAP-70, Her2/ErbB2, Akt, Raf-1, Hif-1a, hormone receptors, survivin, mutant p53, and hTERT [2]. Their role in the folding and maturation of various client proteins, as well as the rematuration of misfolded proteins, makes them potential targets for many diseases ranging from the disruption of multiple signaling pathways associated with cancer [2,3] to the clearance of protein aggregates in neurodegenerative diseases [4]. Current HSP90 inhibitors are categorized into several classes based on distinct modes of inhibition like (i) blockade of ATP binding, (ii) disruption of co-chaperone/HSP90 interactions, (iii) antagonism of client/HSP90 associations and (iv) interference with post-translational modifications of HSP90 [5]. The ATPase activity of HSP90 drives the chaperone cycle and directs binding, induction of the active conformation and release of its client proteins. 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 [6]. 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 [7]. Recently, three synthetic HSP90 inhibitors, with improved pharmacologic profile, have been developed with diverse chemical scaffolds [8]. These inhibitors are being studied for range of cancers in different clinical trials. Currently there are

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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 (Supplementary Table 3). 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 this study as they have improved pharmacologic profile when compared to 17-AAG [9,10]. Pharmacophore and docking models were generated using the above three inhibitors, which was further used to discover novel HSP90 inhibitors in HSP90-over expressing SKBr3 cells.

2. Materials and methods

2.1. Common pharmacophore hypotheses

Common feature pharmacophore hypotheses were generated using a set of three HSP90 inhibitors (1–3, Fig. 2). The structures and conformations of the three compounds were built within Catalyst (Accelrys, Inc.) [11]. The Poling algorithm implemented within Catalyst was used to generate conformations for all of the compounds. For each compound, possible diverse sets of conformations were generated over a 20 kcal/mol range using the BEST flexible conformation generation option available in Catalyst. The HipHop module in Catalyst was used to evaluate a collection of conformational models of molecules and to identify configurations of features (pharmacophore) common to these molecules. The top-ranking pharmacophores are expected to identify the hypothetical orientation of the active compounds and the common binding features interacting with the target. The chemical features considered in the pharmacophore model generation run were H-bond acceptor (HBA), H-bond donor (HBD), hydrophobic (HYA) and ring aromatic (RA) features. HipHop was set to consider these features in the generation of the pharmacophore hypotheses.

2.2. Database search

The representative common pharmacophore hypothesis (Hypo1) was used as a search query to retrieve compounds with novel structural scaffolds and desired chemical features from a multi-conformer Catalyst-formatted database consisting of ~1 million compounds in GVK BIO in-house database [12]. The Fast Flexible Search Databases/Spread Sheets method in Catalyst was used to search the database.

2.3. Glide docking studies

Crystal structure of HSP90 (PDB ID: 2VCI) was used for the docking studies. The protein 3D structure was downloaded from the protein databank (PDB) [13]. The hydrogen atoms were added to the proteins and further minimization was performed using protein preparation wizard. A set of 87 human HSP90 inhibitors was docked into the active site of HSP90 using Glide docking program. The HSP90 grid boxes were defined by the center of the bound inhibitors of the proteins. The enclosing box and binding box dimensions were fixed to 14 Å and 10 Å, respectively. The top 20 poses were collected for each compound. Docking poses were energy minimized using the OPLS-2001 force field. The best pose was selected based on Glide score and the favorable interactions formed between the compound and amino acid residues of the HSP90 active site.

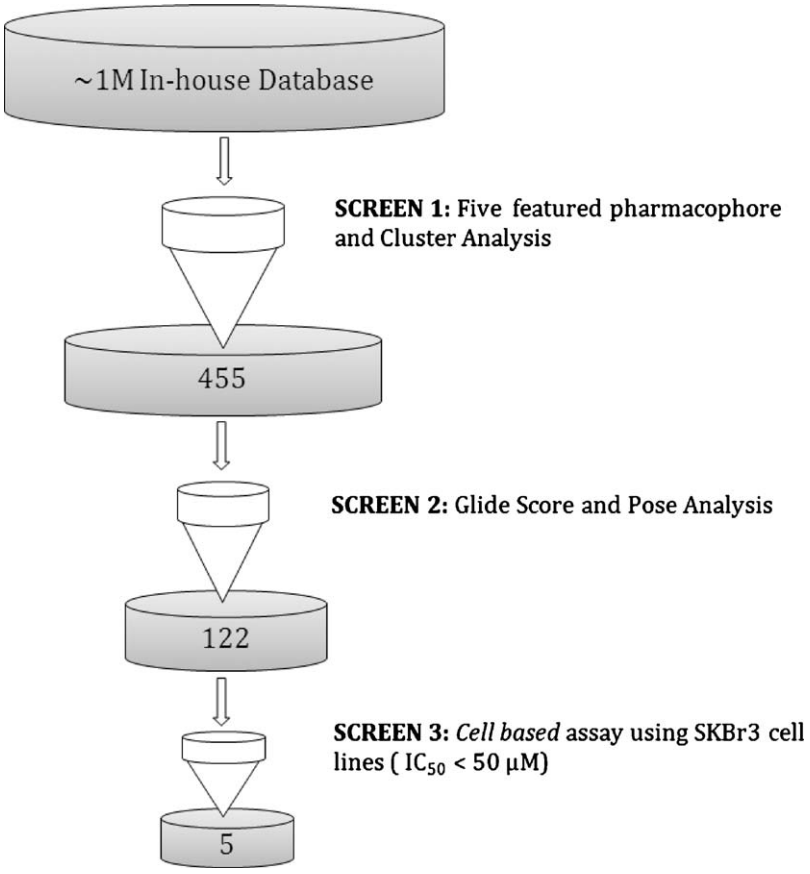


Fig. 1. In silico screening protocol implemented in the discovery of HSP90 inhibitors.

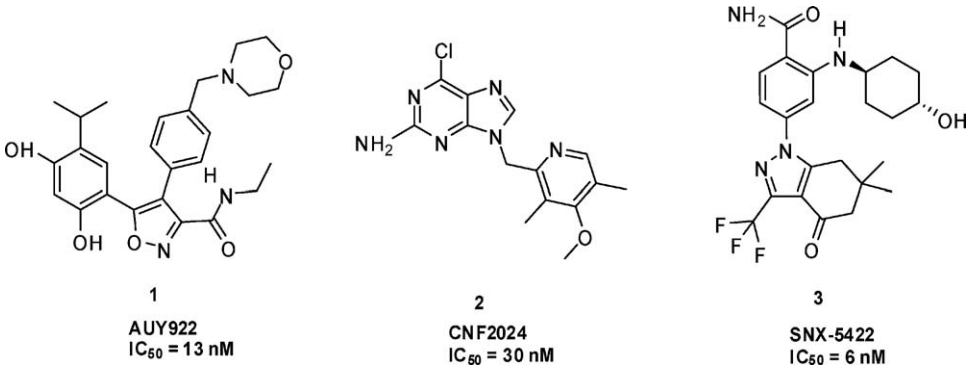


Fig. 2. Chemical structure of synthetic small-molecule HSP90 inhibitors which are currently in clinical evaluation, training set compounds (**1**–**3**), used to generate qualitative pharmacophore models.

2.4. Cell culture

The SKBr3 cell line was obtained from American Type Culture Collection (Manassas, VA) and was used for cytotoxicity assays. Cells were maintained as monolayer cultures in RPMI 1640 and supplemented with 10% fetal bovine serum (Gemini-Bioproducts, Woodland, CA) and 2 mM L-glutamine at 37 °C in a humidified atmosphere of 5% CO_2. To remove the adherent cells from the flask for passaging and counting, cells were washed with PBS without calcium or magnesium, incubated with a small volume of 0.25% trypsin-EDTA solution (Sigma–Aldrich, St. Louis, MO) for 5–10 min, and washed with culture medium and centrifuged. All experiments were done using cells in exponential growth phase. A 10 mM stock solution of each compound was prepared in DMSO and stored at −80 °C. Further dilutions were freshly made in media.

2.5. Cytotoxicity assay

Cytotoxicity was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 8000 cells were seeded in 96-well microtiter plates and allowed to attach overnight. Cells were subsequently treated with continuous exposure to the corresponding drugs for 72 h. A MTT solution (at a final concentration of 0.3 mg/mL) was added to each well and cells were incubated for 4 h at 37 °C. After removal of the medium, DMSO was added and the absorbance was read at 570 nm. The IC_{50} values were then determined for each drug by plotting the log value of drug concentration versus percent inhibition of cell growth.

3. Results and discussion

3.1. Design of HSP90 inhibitors

We utilized three synthetic small-molecule HSP90 inhibitors, currently in clinical evaluation, 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. The overall work flow of molecular modeling and biological assay to identify novel HSP90 inhibitors is schematically shown in Fig. 1.

3.2. Generation of common feature pharmacophore models

In the screen 1, the qualitative pharmacophore model has been used as a query to retrieve the compounds from the database [14]. The HipHop algorithm in the Catalyst software package was applied to generate a common feature pharmacophore model using a training set of 3 synthetic small-molecule HSP90 inhibitors, each with comparable binding affinities to HSP90 (0.002–0.09 μM) (Fig. 2) [15–18]. The training set compounds were chemically diverse set and were expected to have a similar binding mode in the active site of HSP90. The pharmacophoric features were selected based on (1) the structural and chemical features of the training set inhibitors, (2) the architecture of HSP90 active site, and (3) the critical interactions observed between the crystal ligand and the key residues present in the HSP90 active site (PDB 2VCI). The features considered in the pharmacophore model generation were H-bond donor (HBD), H-bond acceptor (HBA), hydrophobic (HYA) features and ring aromatic (RA) feature. Each HipHop run generated 10 optimal pharmacophoric hypotheses, yielding 100 models from 10 automatic runs. All these pharmacophores were validated with internal and external data sets.

3.3. Validation of common feature pharmacophore models and database screening

As an internal validation of the pharmacophore models, the training set compounds (Fig. 2) were mapped onto each pharmacophore. Based on the internal validation of these 100 pharmacophores, the best pharmacophore hypothesis (Hypo1) consisted of one hydrogen bond donor, one hydrogen bond acceptor and three hydrophobic features confirming a good agreement between critical chemical features of these three compounds and the pharmacophore models (Fig. 3). As an external validation and also to evaluate the discriminative ability of these pharmacophores in the separation of active compounds from inactive compounds, the representative pharmacophores were further used as 3D queries to search a database of known HSP90 inhibitors. This database has a total of 87 compounds with a wide range of activity profiles against the HSP90. The results were analyzed using a set of parameters such as hit list (H_t), number of active percent of yields (%Y), percent ratio of actives in the hit list (%A), enrichment factor (E), false negatives, false positives, and goodness of hit score (GH) (Table 1) [19]. Hypo1 succeeded in the retrieval of 75% of the active compounds from the test set. In addition, the pharmacophore also showed an enrichment factor of 1.73 and a GH score of 0.61 indicating that the quality of the model is acceptable. The external validation also confirmed that the Hypo1 had performed better than all the other pharmacophores. The obtained pharmacophore model (Hypo1) was compared with the crystal ligand interactions present in HSP90 Co-crystal. The common HBD feature of the pharmacophore models complimented the hydrogen-bonding interaction involving the resorcinol moiety and the carboxylate group of the residue Asp93. The one HBA feature mapped to the interaction between the O atom of the

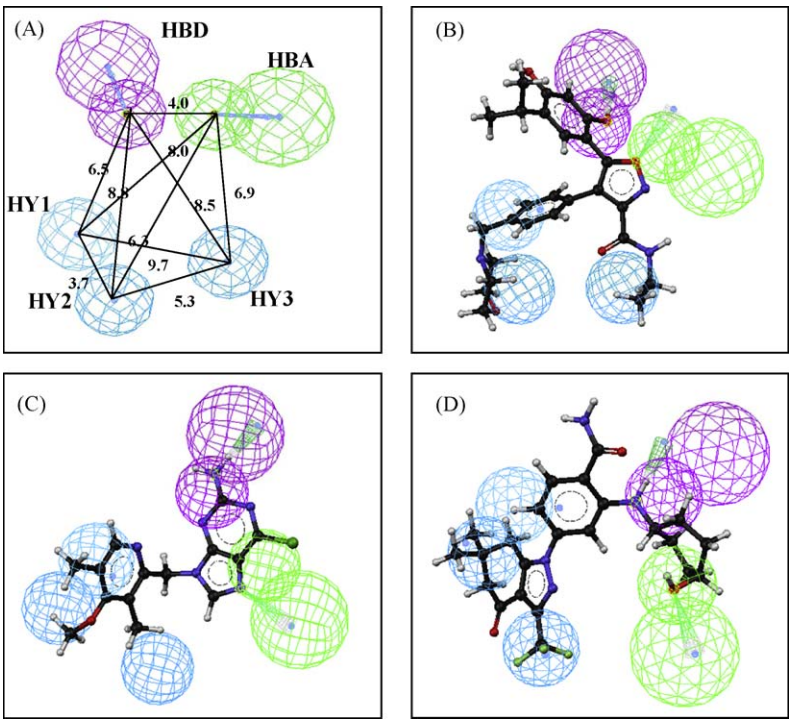


Fig. 3. (A) Three-dimensional arrangement of pharmacophore features in the qualitative pharmacophore model (HipHop1). Pharmacophore features are: H-bond acceptor (HBA), H-bond donor (HBD) and hydrophobic (HY 1–3). (B) HipHop1 is mapped onto compound **1**, (C) mapping of HipHop1 onto compound **2** and (D) mapping of HipHop1 onto compound **3**.

isoxazole ring with the water molecules and hydrophobic interactions were also seen with LYS58, ILE96, LEU107 THR109 and GLY135. Based on the Hypo1's superior ability to select potent inhibitors and its discriminating power to distinguish actives from inactives, 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. The initial screening of Hypo1 yielded 5340 compounds and further cluster analysis of these hits corresponded to 455 unique cluster representatives. These hits were further analyzed using docking studies.

3.4. Docking studies

In the screen 2, the docking method has been implemented to further filter the pharmacophore hits. Docking studies were performed for HSP90 inhibitors into the active site of 3D structure of HSP90 (PDB ID: 2VCI) using Glide program [20,21]. The Co-crystal complex of HSP90 (2VCI) with crystal ligand (Fig. 2, CNF-2024) is used to identify the binding orientations and the reliability of docking method to predict the bioactive conformation [22]. Crystal ligand (Fig. 2, AUY922) was docked into the active site of HSP90 and the docked conformation with the lowest docking energy was selected as the most probable binding conformation. The superimposition of Glide predicted conformation of AUY922 with its crystal conformation is shown in Fig. 4A. The root mean-square deviation (RMSD) between these two conformations was found to be 1.06 Å, suggesting a high docking reliability of Glide in terms of reproducing the experimentally observed binding mode for HSP90 inhibitors. 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 was calculated between dock score and the pIC_{50} using linear regression analysis method. An acceptable correlation coefficient (r) of 0.81 was obtained between experimental pIC_{50} and docking energy. This correlation proved that the binding modes of the HSP90 inhibitors are reliable and this is 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, formed good interactions with the key residues in the protein and showed high dock score. The bound conformation of compound **4** (Cyan) and Crystal ligand (Yellow) on the active site of HSP90 is shown in Fig. 4B. Based on the docking score, 122 compounds were purchased from chemical vendors and tested against SKBr3 cell lines, which are shown to over express HSP90 [23]. Of the 122 compounds tested, 5 compounds inhibited cell growth with an IC_{50} value ranging from 2 μM to 32 μM (Table 2). Compounds **4** and **5** inhibited the proliferation of SKBr3 cells with IC_{50} values of 2.05 μM and 7.01 μM, respectively. As envisaged by the pharmacophore model, the 6,7-dimethoxy-1H-quinolin-2-one (compound **4**) and 5-pyridin-3-yl-4H-[1,2,4]triazole-3-thiol ana-

Table 1
Validation of the pharmacophore model using statistical parameters.

Total compounds in database (D)	87
Total number of actives in database (A)	38
Total hits (H_t)	45
Active hits (H_a)	34
% yield of actives	75.56
% ratio of actives in the hit list	89.47
Enrichment factor or enhancement (E)	1.73
False negatives	4
False positives	11
GH score (goodness of hit list)	0.61

$[((H_a/4H_tA)(3A+H_t)) \times (1-((H_t-H_a)/(D-A)))]$; GH score of 0.6–0.7 indicates a good model.

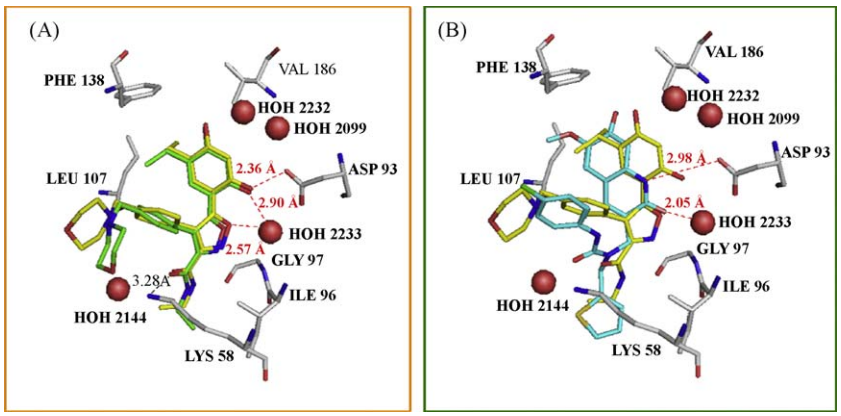
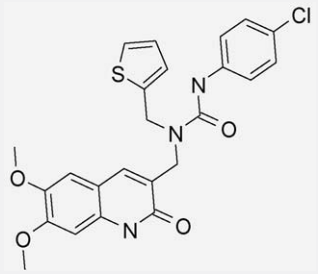
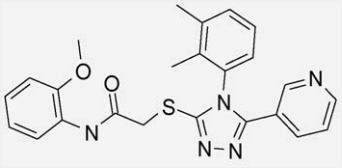
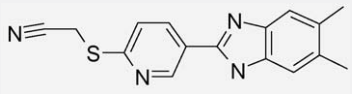
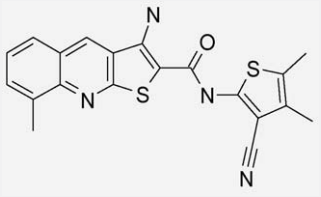
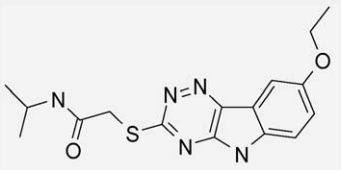


Fig. 4. (A) Overlapping of compound 1 conformation (Crystal, Yellow) with compound **1** conformation (Glide, Green) in the active site of HSP90. (B) Overlapping of active compound **4** conformation (Cyan) with compound **1** conformation (Crystal, Yellow) within the active site of HSP90.

Table 2
Cytotoxicity of pharmacophore hits against HSP90-over expressing SKBr3 cell line.

Compound no.	Structure	Cytotoxicity in HSP90-over expressing SKBr3 cell line IC_{50} (μM)
4		2.05
5		7.01
6		25.27
7		26.97
8		31.27

logue (compound **5**) exhibited enhanced inhibitory activity as compared to the compounds **6**, **7** and **8** against the proliferation of SKBr3 cells. Further chemical optimization is required to better understand the key residues for activity and selectivity.

4. Conclusions

Several structurally diverse compounds possessing growth inhibitory potency against HSP90 over expressing cancer cells

were identified using pharmacophore, cluster analysis and docking studies. The pharmacophore and docking models were generated and validated utilizing a set of known HSP90 inhibitors. These compounds bearing amenable chemical and structural features are potential leads for drug design strategies targeting HSP90. In conclusion, it has been shown that, modification of typical pharmacophore and combination of docking with pharmacophore based virtual screening can improve the activity of hits. These hits are under optimization for further drug development to get more druggable lead.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmgm.2009.11.002.

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