Letters

Integration of Ligand and Structure-Based Virtual Screening for the Identification of the First Dual Targeting Agent for Heat Shock Protein 90 (Hsp90) and Tubulin

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Abstract: We describe the discovery of a novel indazole-based scaffold that represents the "first-in-class" dual Hsp90/tubulin binding compound. Individual known ligands for both targets shared similar 3′,4′,5′-trimethoxyphenyl cores, and from this it was hypothesized that application of an integrated ligand and structure-based virtual screening (VS) workflow could yield a single scaffold with dual binding affinity. Following validation of the VS protocol, we successfully identified a novel dual inhibitor, sourced from a commercial screening collection of 160 000 compounds.

Heat shock protein 90 (Hsp90) functions to protect cells when stressed by elevated temperatures, and in unstressed conditions it assists in regulation of cell signaling, folding, transport, maintenance, and degradation of proteins. Its involvement in multiple signaling pathways on which cancer cells depend for growth and survival makes it a valuable anticancer target.[1,2]

The most well-known inhibitor of Hsp90, 17-(alkylamino)-17-demethoxygeldanamycin (17AAG), is currently in phase II clinical trials for the treatment of cancer,[3] with others such as the purine scaffold (CNF-2024),[4] the isoxazole derivative (NVP-AUY922),[5] the carbazol-4-one benzamide derivative (SNX-5422)[6] following suit. Structure-based drug design (SBDD) has played a prominent role in aiding this process, with virtual screening (VS[a]) also coming to the forefront in their discovery. Barril et al.[7,8] first described docking-based VS of Hsp90 leading to the discovery of novel classes of purine-base and adenine-based inhibitors that compete for the N-terminal ATP binding site. Several examples of application of VS to this end have since been published with docking-based platforms being deployed,[8−10] delivering not only new chemotypes but also highlighting the impact that treatments of receptor flexibility and water molecules have on the docking process. The structural diversity of Hsp90 inhibitors is considerable,[6] with levels of inhibition being related to the ability of each inhibitor to modulate the conformational dynamics of the protein.[11]

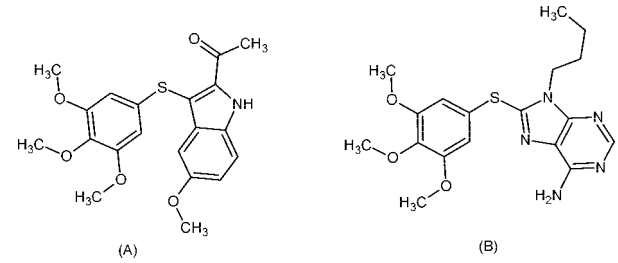

Figure 1. Common 3′,4′,5′-trimethoxyphenyl pharmacophore for scaffolds: (A) tubulin inhibitor 3-arylthioindole;[14] (B) PU3 analogue Hsp90 inhibitor.[12]

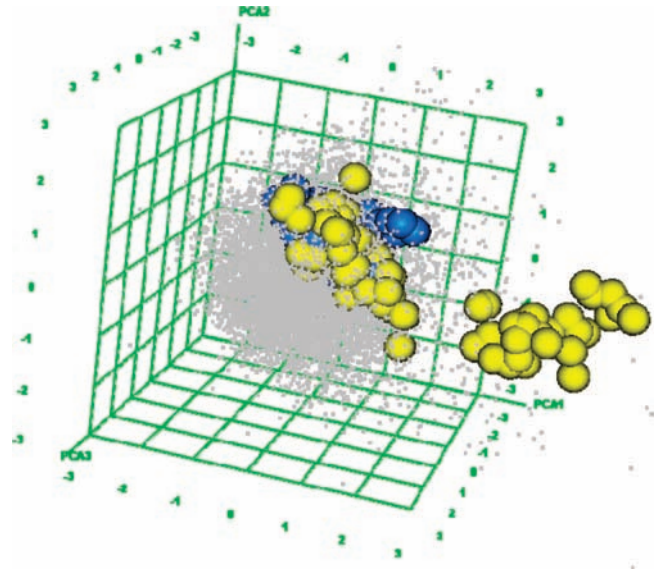

Figure 2. Hsp90 and tubulin binders overlap in the medicinal chemistry space: gray, med chem space; yellow, Hsp90; blue, antitubulin.

A recent structure of a Hsp90 inhibitor[12] was published containing a 3′,4′,5′-trimethoxyphenyl moiety, observed by many groups as being important for binding of ligands in the colchicine binding site.[13] Figure 1 illustrates the commonalities between the potent, 3-arylthioindole tubulin-binding compound[14] and a purine based analogue (PU3) that binds Hsp90.

It was hypothesized that building from this structural commonality it might be possible to identify a single molecule that would inhibit both targets, using VS techniques. To advance this hypothesis, the relative regions that Hsp90 and colchicine-site actives occupy within medicinal chemistry space were mapped. Principal component analysis (PCA) was carried out as previously published by our group,[15] with multidimensional selection of 165 descriptors calculated in MOEv2007.09.[16] PCA allows us to reduce dimensionality, preserving the major differences and similarities between molecules but removing redundancy between them. In Figure 2, we have reduced 165-dimensional space into three-dimensional space for a database containing 129 Hsp90/49 tubulin actives selected from literature and a set of 10 000 randomly selected molecules representing generic medicinal chemistry space extracted from the ZINC database.[17]

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[a] Abbreviations: VS, virtual screening; LBDD, ligand-based drug design; SBDD, structure-based drug design; PCA, principal component analysis; AUC, area under the curve; rmsd, root mean square deviation.

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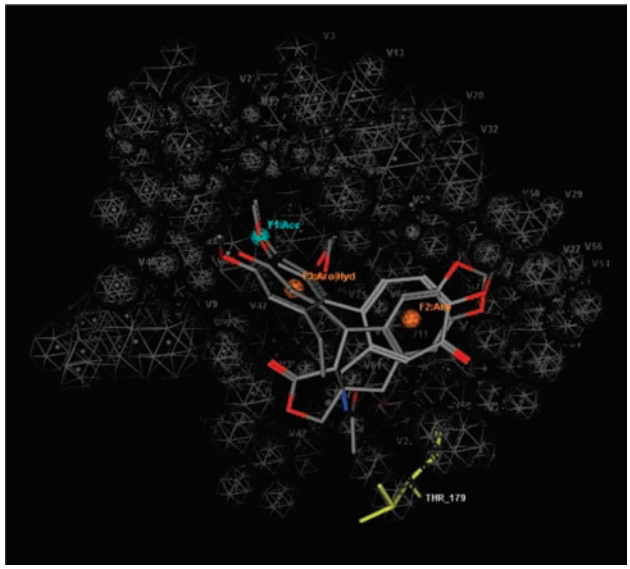

Figure 3. Pharmacophore of "colchicine" binding site in tubulin: cyan, acceptor; orange, aromatic; gray, volume constraints represented as unified surface; yellow, residue Thr179.

Figure 2 demonstrates that there is overlap and chemical space clustering between many of the known Hsp90 and tubulin actives. In fact, those Hsp90 compounds that lie outside the PCA axes are compounds containing ansamycin cores that have a far greater molecular weight compared with the tubulin set. To investigate these commonalities further, the recently published "target-fishing" technique[18] was implemented in this work through construction of a Bayesian model using Pipeline Pilot,[19] designed to identify molecules that bind the ATP binding site of Hsp90. Our Bayesian model was built using a set of 26 active ligands (see Supporting Information) and was tested by its ability to discriminate Hsp90 actives from a set of 5000 decoy molecules, carefully selected to ensure any bias was removed. Once the model was optimized, it was applied to screen the WOMBAT database[20] to identify molecules structurally and chemically similar to Hsp90 actives, whose targets and mechanisms were robustly annotated. Importantly, 14% of compounds that bound to the tubulin colchicine binding site were deemed to be structurally related to those of Hsp90, reflecting a degree of molecular structural overlap. These initial investigations supported our decision to utilize a VS approach in the search for a novel dual inhibitor of both targets. On the basis of available structural and ligand data, it was decided to employ docking for Hsp90 and pharmacophore matching for tubulin investigations. Full details of all computational procedures are described in the Supporting Information. First, a docking regime across all available X-ray structures of Hsp90 (33 X-rays) with bound ligands was carried out. As previously detailed by Barril et al.,[21] using multiple receptor conformations of Hsp90 does not necessarily translate into an improvement in "hit" rates, but it did inform selection of our choice of docking methodology. All molecules were enumerated from 2D to 3D using OMEGAv2.2.1[22] after ionization at pH 7.4 with Pipeline Pilot.[23] Two different conformational sets (generation of 10 and 50 conformers) were assessed to investigate any improvements to the docking/scoring process as in our previous work[24] and to investigate the use of specific software in a target specific manner.[25] Selection criteria for the final individual X-ray structure to employ in the docking process were based on prioritization, diversity, and total number of actives retrieved using the DUD Hsp90 validation set.[26] As observed by Barril

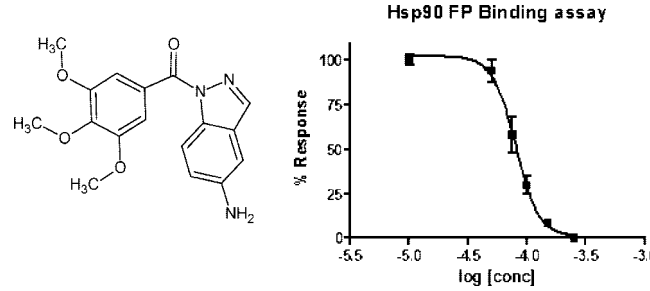

Figure 4. Structure of **1** and Hsp90 FP ATP-site binding assay with **1**.

et al. previously, large variations were observed in hit rates, dependent on our receptor choice, which upon further investigation could be for the most part attributed to movement of Lys44. It is possible that inclusion of flexibility of this residue would enhance the docking procedure, and future efforts will be focused in this area rather than docking to multiple X-ray structures. In the current study however, docking in PDB entry 1OSF with FREDv2.2.3[27] and scoring with Chemgauss3 was sufficient. Ten conformations of all molecules were enumerated, as no additional benefit was observed at this stage upon enumeration of 50 conformers per molecule.

Molecules that bind the ATP pocket of Hsp90 are known to exhibit key H-bonding contacts with Thr184 and Asp93, and it was considered beneficial to introduce a distance constraint to ensure selection of only those binding modes that satisfy the donor−acceptor pharmacophore between all ligands and Asp93 and/or Thr184. An area under the curve (AUC) of 0.998 was achieved using this methodology, indicating utility of the model in distinguishing actives from decoys. All molecules from subsequent VS runs were passed through this docking/scoring protocol and retained for calculation of "fit" to our tubulin pharmacophore outlined next. Second, a ligand-based pharmacophore was designed using initially information from eight structurally diverse ligands (see Supporting Information) that bind to the colchicine binding site of tubulin. Included in the set were three ligands extracted from PDB entries 1SA0, 1SA1, and 1Z2B. These three ligands were rigidly aligned and all remaining ligands flexibly superposed utilizing the alignment module in MOE.2007.09. Features for the pharmacophore were generated based on a consensus of the aligned structures resulting in three common features. After a subsequent test of the pharmacophore, using a "haystack" of actives and decoys, it was deemed necessary to add receptor-based information to improve the robustness of the model due to the simplicity of the donor/acceptor/aromatic features of the initial ligand-based pharmacophore. Figure 3 illustrates the receptor-based pharmacophore, with colchicine and podophyllotoxin superimposed by backbone from their respective X-ray structures (PDB entries 1SA1 and 1SA0). The consensus pharmacophore generated from the ligand data is shown with the three features common to all ligands. These donor/acceptor/aromatic features were retained, and volume features were added accordingly representing positioning of active-site residues (e.g., Thr179 in Figure 3) and upon completion provided an accurate "mold" of the active site.

Following addition of a volume constraint, it was possible to clearly discriminate between actives and decoys in the "haystack" (AUC of 0.891; see Supporting Information). The validated VS protocol was now applied to the SPECS commercial compound library (∼160 000 unique compounds) to identify compounds that hit both targets. The library was first passed through the Hsp90 Bayesian model, with only a total of

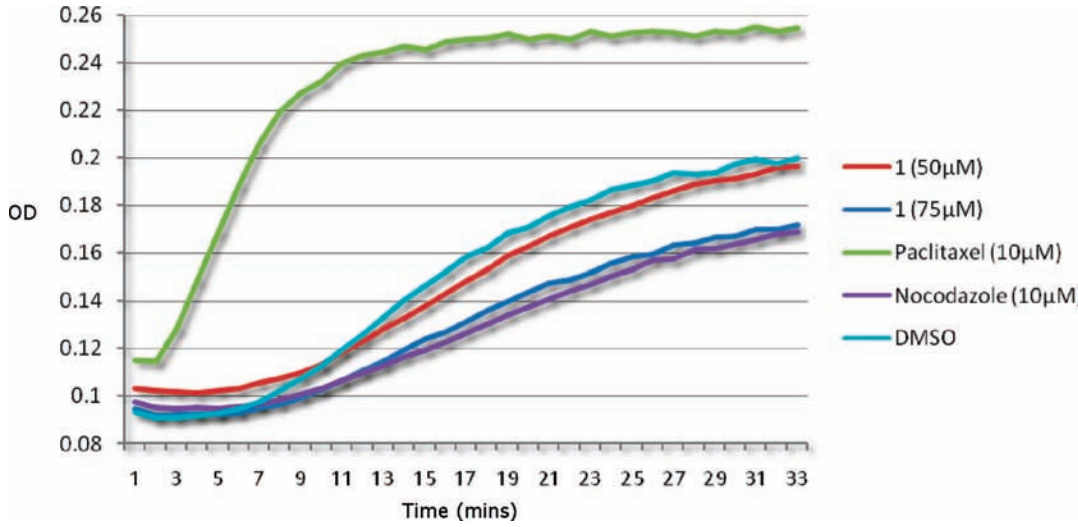

Figure 5. Polymerization of tubulin: (A4) **1** at 75 μM; (B4) **1** at 50 μM; (C4) paclitaxel at 10 μM; (D4) nocodazole at 10 μM; (E4) DMSO.

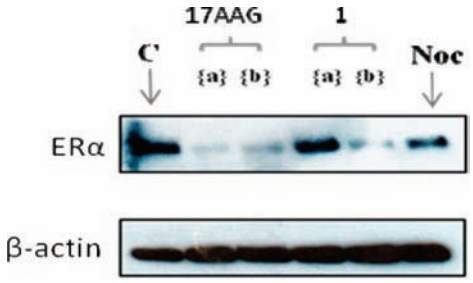

Figure 6. Western blot analysis of **1** induced ERα degradation. MCF-7 cells were treated with vehicle (C, 0.5% DMSO), 17-AAG ((a) 1 μM and (b) 10 μM), **1** ((a) 80 μM and (b) 200 μM), and nocodazole (Noc, 1 μM) for 24 h. Whole cell lysates were prepared and analyzed for estrogen receptor α (ERα) expression by way of Western blotting (β-actin used as a loading control).

513 compounds remaining. Those compounds were subsequently passed to the Hsp90 docking/scoring procedure, and all successfully docked compounds progressed to the colchicine active site pharmacophore. From the prioritized compounds (51 in total), 20 were visually selected and subsequently purchased and tested for their ability to bind Hsp90 and to also depolymerize tubulin. Hsp90 binding was measured by fluorescence polarization as described previously by Howes et al.[28] From the 20 ligands assessed, two displayed modest binding affinity to Hsp90 and were carried forward for testing in a tubulin polymerization assay. One compound (ranked 40th by root mean square deviation (rmsd) after passing tubulin pharmacophore) displayed binding to the ATP site of Hsp90 with an IC_{50} of 80.76 $\pm$ 0.0235 μM ($n = 3$) (Figure 4) and also the ability to depolymerize tubulin. The ligand 1-[(3,4,5-trimethoxyphenyl)-carbonyl]-1*H*-indazol-5-amine **1** (MDG892) (Figure 4) exhibited a dose−response depolymerizing effect with 75 μM being equivalent to 10 μM nocodazole treatment ($n = 2$) (Figure 5).

A cell based assay was used to test the ability of **1** to interact with the Hsp90 ATP-binding site and induce proteosomal degradation of Hsp90 client proteins. MCF-7 cells were treated with **1**, and whole cell lysates were analyzed for degradation of Hsp90 client protein, estrogen receptor α (ERα), by way of Western blotting (Figure 6). Treatment with 200 μM **1** induced significant degradation of ERα when compared to vehicle control. 17-AAG, a known Hsp90 ATP-binding site ligand, also induces ERα degradation at lower concentrations. Treatment with nocodazole, a ligand of the tubulin colchicine binding site, did not induce any significant level of degradation of ERα. These data indicate that **1** can induce Hsp90 client protein degradation and that this effect is mediated through its ability to bind directly to the Hsp90 ATP-binding site and not related to its affinity for the colchicine site.

To carefully analyze the binding modes of **1** in Hsp90 and tubulin, a rigid docking procedure, FRED followed by reoptimization of the binding pocket and ligand simultaneously using LigX (MOEv2007.09) was carried out. With examination of the predicted the binding mode of **1** in the ATP pocket of Hsp90 first, it is clear that several key interactions regularly observed with Hsp90 inhibitors are likely formed, such as hydrogen bonding with Asp93 and two H-bonds with Lys112. No direct bonding to Thr184 was observed; however, hydrogen bonding with interstitial water was predicted, mirroring that observed with other Hsp90 inhibitors (Figure 7). It has been reported that for optimal inhibition of Hsp90, H-bonding to Thr184 is thought be essential, and this will be investigated in further studies.

The binding orientation of **1** in the colchicine binding site is predicted tobe similar to known inhibitors with the trimethoxy moiety occupying the same position observed with all colchicine competing compounds (e.g., combretastatin A-4); however, no interaction with Thr179 is observed as is generally present. Instead, a hydrogen-bonding interaction with Ser178 (3 Å) is predicted to form and helps to secure the ligand in the active site. It is more likely that replacement of the indazole core with 3-arylindole would significantly enhance tubulin binding, as the indole nitrogen would be free and hydrogen-bond with Thr179 without altering its Hsp90 activity. We are currently synthesizing alternative scaffolds to confirm this theory. Rotation about the ketone linker appears crucial to the binding of this compound to both receptors simultaneously allowing the trimethoxy moiety to position itself correctly in both cases.

In conclusion, we have developed and validated a novel integrated ligand-based and structure-based VS workflow designed to retrieve dual Hsp90/tubulin binding molecules, consisting of a docking/scoring protocol and receptor-based pharmacophore utilized sequentially. Application of these modules as a VS workflow when applied to the Specs database of ~160 000 compounds facilitated retrieval of a "first-in-class" novel compound that is active as intended, binding at Hsp90 and tubulin. Implementing "target-fishing" techniques afforded useful data to suggest further investigation of the possibility of targeting both proteins simultaneously with a single molecule.

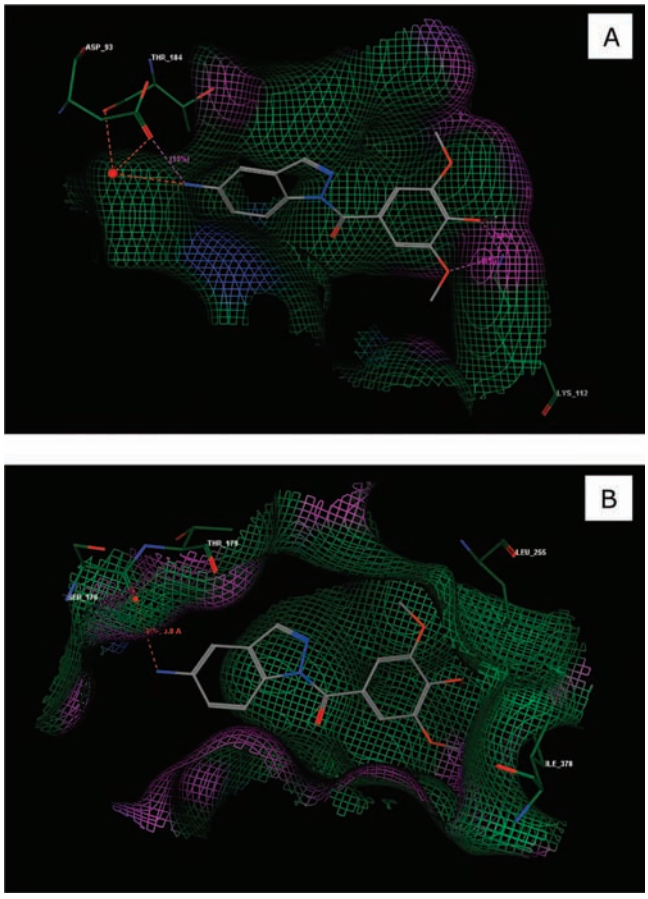

Figure 7. **1** docked in active site of Hsp90 (A) and active site of colchicine (B).

It is envisaged that application of this workflow in a target-specific manner will lead to the discovery of more dual targeting molecules for different therapeutic targets.

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Supporting Information Available: Details of all computational and biological procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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