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Structure-based discovery of a new class of Hsp90 inhibitors

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Abstract—Docking-based virtual screening identified 1-(2-phenol)-2-naphthol compounds as a new class of Hsp90 inhibitors of low to sub-micromolar potency. Here we report the binding affinities and cellular activities of several members of this class. A high resolution crystal structure of the most potent compound reveals its binding mode in the ATP binding site of Hsp90, providing a rationale for the observed activity of the series and suggesting strategies for developing compounds with improved properties.

Many of the client proteins of the molecular chaperone Hsp90 are involved in the onset and progression of cancer, making it a very attractive oncology target.[1] The N-terminal domain of Hsp90 (Nt-Hsp90) binds ATP, which drives the chaperone activity of the protein. Therefore, binding of ligands to this site results in Hsp90 inhibition and therapeutic opportunities. The natural products geldanamycin (**1c**) and radiciol (**2**) were the first to be identified as inhibitors of Hsp90.[2,3] The geldanamycin derived inhibitor 17-AAG (**1b**) has entered phase I clinical trials and initial results are encouraging, providing proof of principle for Hsp90 inhibitors as cancer therapeutics.[4,5] However, 17-AAG has several potential limitations including poor solubility, limited bioavailability, toxicity and extensive metabolism.[6] These issues and the inherent chemical complexity of these compounds have led to significant efforts to identify small molecule inhibitors of Hsp90.[7] New classes of compounds have been discovered by synthetic modification of the natural substrate of Hsp90[8,9] and by high throughput screening of chemical libraries,[10,11] leading to purines such as PU24FCl (**3**) and dihydroxyphenylpyrazoles such as G3129 (**4a**) and CCT018159 (**4b**) (Fig. 1). The Nt-Hsp90 domain is amenable to X-ray crystallographic analysis and structures of human Nt-Hsp90 in complex with purine and resorcinol-based inhibitors have been successfully applied to rationalize structure–activity relationships and to guide the optimization of these series[12–14] to produce compounds such as VER-49009 (**4c**).

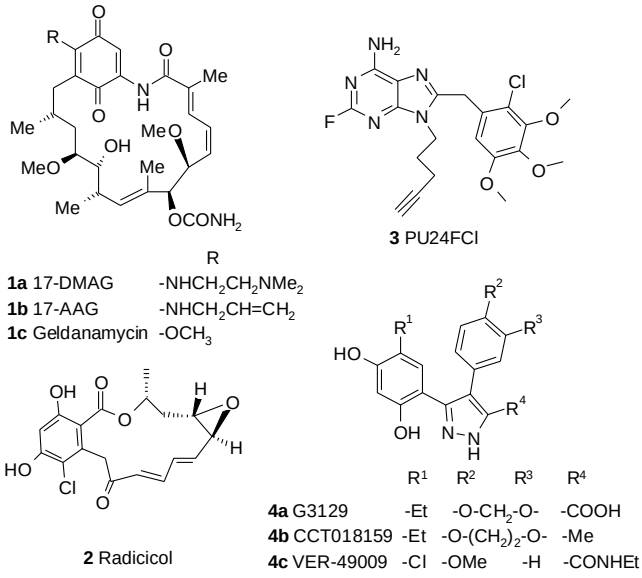

Figure 1. Small molecule inhibitors of Hsp90.

There has recently been increasing success in using molecular docking methods[15] in the discovery of new chemical entities that bind to structures of therapeutic targets.[16,17] Here we report how this approach led to the discovery of 1-(2-phenol)-2-naphthol as a new class of Hsp90 inhibitors. The initial set of hits found by

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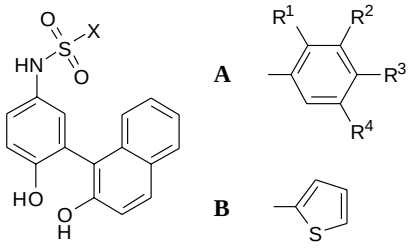
means of docking-based virtual screening were augmented with closely related compounds found in commercial catalogues, providing valuable initial SAR. Moreover, the crystal structure of the most potent member of the series has been obtained, providing insights into the most promising optimization avenues for this series.

In order to identify new classes of Hsp90 inhibitors, we used a virtual screening protocol, which has been published elsewhere[16] and consists of four main steps. The first is definition of the binding cavity. The shape and size of the ATP binding site are altered upon binding of purines,[12] so two structures of Nt-Hsp90 corresponding to the open and helical forms were used (PDB codes 1YET[18] and 1UY6,[12] respectively). Three interstitial water molecules consistently found around the carboxylic group of Asp93 were kept as part of the receptor. The second step is preparation of the database of small molecules to be docked. A virtual library of 0.7 million compounds was selected from the 3.5 million commercially available compounds in our proprietary catalogue rCat,[19] on the basis of drug-likeness,[20] removal of reactive groups and vendor delivery timelines. The program CORINA,[21] version 3.00 was used to convert the docking library from 2D to 3D. The third step used the program rDock (Morley et al., unpublished results) to screen the docking library against Nt-Hsp90 and identify 9000 highest-scoring compounds for further analysis. The fourth and final step is postfiltering the compounds. All the Hsp90 inhibitors for which a structure of the complex with the protein is available form a hydrogen bond with one carboxylic oxygen of Asp93 and accept a second hydrogen bond from an interstitial water molecule. A Perl script was used to retain only the predicted binding modes that satisfy this donor–acceptor pharmacophore. These compounds were clustered in families based on the scaffold they use to satisfy the pharmacophore; the number of members in a few over-represented families was reduced using two-dimensional three-point pharmacophore fingerprints (as implemented in MOE; Chemical Computing Group Inc., Montreal) to maximize chemical diversity within the family. This resulted in 1000 compounds selected for purchase, of which 719 were actually available from commercial suppliers and were assayed in a malachite green assay.[22] This led to the discovery of several chemical families, including resorcinol–pyrazoles similar to **4a** and **4b**, the phenol–naphtholes herein described and others. Out of five compounds of general formula 1-(2-phenol)-2-naphthol present in the screening library, three were identified as hits (greater than 50% inhibition) in the primary assay,[22] of which two (compounds **7** and **13**) had low to sub-micromolar activity. The central scaffold common to both compounds was used to perform substructural searches in commercial chemical catalogues, from where seven compounds (**5**, **6**, and **8**–**12**) were purchased and assayed, all of which turned out to be active. The IC_{50} values (determined with a fluorescent polarization assay[23]) are provided in Table 1 for all these compounds.

The cellular activity of the three compounds with higher binding affinity (**7**, **11** and **13**) was assessed using a growth inhibition assay on human colon cancer cell line HCT116 and is also reported in Table 1. In all cases, the GI_{50} is significantly greater than the IC_{50}, by a factor of 13 (**7**), 41 (**11**) or 100 (**13**), suggesting that the compounds are not well absorbed by the cell. Although somewhat disappointing, the poor GI_{50}/IC_{50} ratio of these compounds (particularly **11** and **13**) is not unexpected because they have not been optimized for cellular potency. Pharmacodynamic marker changes in human breast cancer BT474 cells after 48 h of exposure to compound **11** at a concentration equivalent to the GI_{50}

Table 1. Binding and growth inhibition assay results for compounds **5**–**13**



Compound	X	R^1	R^2	R^3	R^4	FP enzyme IC_{50}, μM[a]	Cell growth inhibition, μM[a]
5	A	H	H	H	H	3.3	n.d.
6	A	H	H	F	H	8.8	n.d.
7	A	H	H	Cl	H	2.2	27.8
8	A	H	H	Br	H	6.1	n.d.
9	A	H	H	Et	H	6.6	n.d.
10	A	H	H	*t*-Bu	H	26.4	n.d.
11	A	Cl	H	H	Cl	0.7	29.0
12	A	Me	H	Me	Me	15.2	n.d.
13	B	—	—	—	—	0.6	59.8
17-AAG						1.27	0.16
Radicicol						0.15	0.13

Activities of 17-AAG and radiciol are provided for comparison.

[a] Values are means of at least two experiments.

(34.9 μM) were examined by Western blotting. A reduction was seen in the cellular levels of CDK4 alongside up-regulation of Hsp70 (data not shown). This is consistent with the mode of action for growth inhibition being through inhibition of Hsp90 activity.

In order to determine the binding mode of this class of compounds, X-ray crystallography experiments were performed as follows: Nt-Hsp90α His-tagged protein was concentrated to 20 mg/ml into a final buffer containing 25 mM Tris, pH 7.5, 0.15 M NaCl and 5 mM DTT. Apo Hsp90α crystals were grown in sitting drops at 4 °C using crystallization conditions containing 25% PEG-ME2K, 0.1 M cacodylate, pH 6.5, and 0.2 M magnesium chloride. Single apo crystals were removed from the crystallization drops and placed in a solution of 4 μl crystallization reservoir plus 0.5 μl of 20 mM compound **11** in 100% DMSO. The crystals were left to soak at 4 °C for approximately 16 h.

The ligand-bound data set was collected at cryo-temperature. Reservoir solution, with the PEG concentration increased to 35%, was used as the cryoprotectant. Data were collected on an in-house RU-H3R rotating anode generator with R-Axis IV++ image plate detector and were subsequently processed using D*Trek (Rigaku/MSC). The crystals belong to space group *P*21212 with unit cell dimensions isomorphous to those of our previous PU3-bound Hsp90α structure[12] (PDB code 1UY6). The structure was solved by isomorphous replacement using the Hsp90α protein model coordinates (with the PU3 ligand and solvent removed) and refined with the program REFMAC5.[24] Twenty cycles of rigid-body restrained refinement were then carried out, followed by model building using the molecular graphics program O.[25] Crystallographic water molecules were added by cycling REFMAC5 with ARP/wARP.[26] Once refinement of the structure was completed (R factor = 19.4; R_{free} = 24.1) the structures were validated using PROCHECK[27] and the SFCHECK and WHATCHECK programs from the CCP4i package.[28]

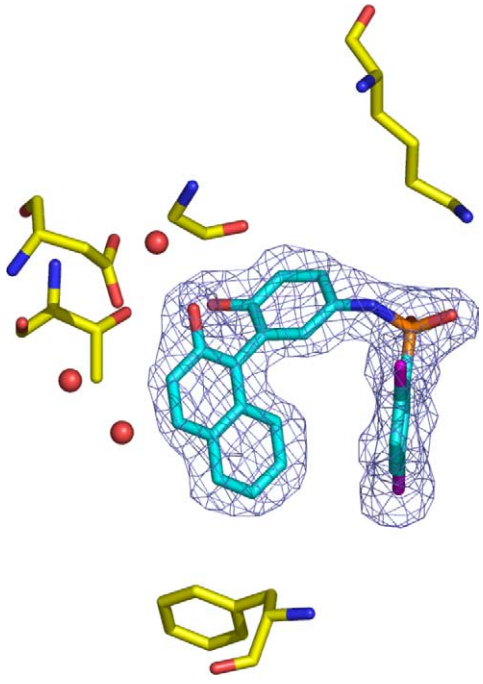


Figure 2. Binding mode of **11** to the ATP binding site of Hsp90. The blue grid depicts the 2fo-fc electron density map countered at a level of 2σ.

Within the crystal there are two monomers in the asymmetric unit, labelled A and B. These are essentially identical in structure (root mean square deviation is 0.50 Å) apart from a difference in the flexible loop between residues 106 and 116. In monomer A, the flexible loop adopts a conformation similar to that of the open form, with Lys112 shifted towards the active site. In monomer B, the flexible loop is almost completely helical. The electron density clearly defines the structure and the ligand binding mode (Fig. 2), which is the same in both monomers (although the ligand only has 80% occupancy in monomer B). Conserved water molecules can also be seen quite clearly within the binding site in both monomers.

The crystal structure of **11** in complex with Hsp90 reveals a binding mode reminiscent of radicicol[29] and other resorcinol-containing compounds.[10,11,14] The substituted ring of the naphthol is located in essentially the same position as the resorcinols (Fig. 3) and the key interaction with Asp93 is provided by a hydroxyl in both inhibitor classes. Ethyl and chloro substitutions of the resorcinol ring have been reported to provide better fitting with the binding site and to be important for activity.[11,14] In our compounds, the unsubstituted naphthol ring appears to play a similar role. The structures of radicicol and CCT018159-like molecules reveal a hydrogen bond acceptor atom (provided by an ester moiety and a pyrazole ring, respectively), in the vicinity of Oγ of Thr184 and an interstitial water molecule. The phenol moiety of compound **11** is at 2.8 and 3.1 Å from the oxygen atoms of these groups, respectively, providing further indication of the importance of these interactions.

In addition, the crystal structure shows that the sulfonamide group does not make any interaction with the protein and merely acts as a linker between the phenol and benzene rings. The di-chloro-phenyl ring of **11** is located at the entrance of the binding site and also does not make any key interactions with the protein. This explains why most substitutions seem to be well tolerated, which is in agreement with the fact that pyrazoles also tolerate a wide range of substituents at position 4,[14] which, as shown in Figure 3, localize in the same region as the phenyl ring. Of all the compounds investigated, **10** is the least active, possibly due to a clash between the bulky *tert*-butyl group and Gly135. On the other hand, the R1 and R4 chlorine atoms of compound **11** make contacts with Asp54 and Leu106, respectively, explaining the improved activity. The sulfur atom of the thiophene ring in compound **13** is likely to make similar favourable contacts.

As shown in Figure 4, the binding mode of compound **7** predicted by docking compares very well with the experimental binding mode observed for compound **11**. As explained above, the key interactions between the pro-

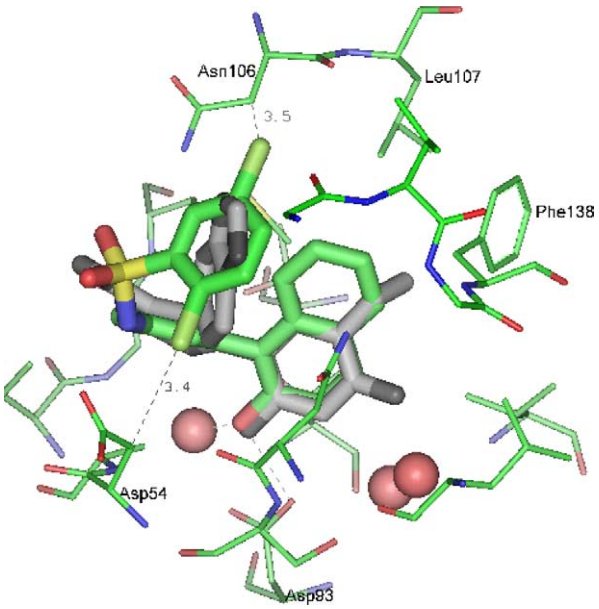


Figure 3. Comparison of the binding mode of **11** (colour-coded by atom) with G3129 (carbon atoms in grey, heteroatoms in black).

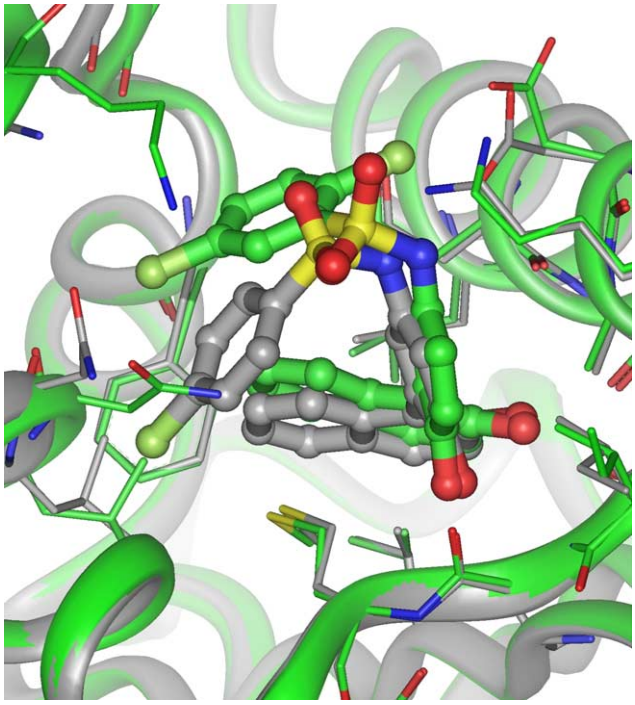


Figure 4. Comparison of the binding mode of **11** (carbon atoms in green) with the predicted binding mode of **7** (carbon atoms in grey).

tein and this series of compounds are mediated by the naphthol and phenol rings. The predicted binding mode for this part of the molecule is within 0.6 Å rmsd to the crystallographic coordinates. In contrast, the chloro-substituted phenyl rings adopt significantly different positions, but this is due to the different conformation of the protein in that region, particularly the side chain of Asn106. This shows that, as long as the key interactions are correctly predicted, our docking protocol tolerates fluctuations in the conformation of the protein.

Beyond rationalization of the SAR, the crystal structure of **11** indicates which areas of the molecule are more suitable for optimization. It is clear that the unsubstituted ring in the naphthol system is not strictly necessary for binding and it could be replaced by other groups provided that (a) favourable lipophilic contacts with Phe138 are maintained, and (b) the twist between the rings (approximately 65°) remains unchanged. This is important to guarantee a correct position of the two phenolic oxygens near Asp93 and Thr184, respectively. Another optimization avenue could be the introduction of large and lipophilic groups able to induce a conformational change in the flexible loop and occupy the hydrophobic pocket used by PU3 and similar compounds.[12] The overlay in Figure 3 also shows that small substituents such as hydroxyl could be tolerated at position 4 of the naphthol ring. Finally, the phenyl sulfonamide could possibly be replaced with a wide variety of chemical groups, including solubilizing elements. Unfortunately, the crystal structure suggests that replacement of any of the metabolically liable phenolic groups is likely to result in a major loss of potency.

In conclusion, a new class of Hsp90 inhibitors has been discovered by means of docking-based virtual screening. Its binding mode to Hsp90 has been determined by X-ray crystallography, enabling us to rationalize the SAR and identify the chemical modifications with greater potential to improve the series. The growth inhibition capacity of these series is sub-optimal, but it seems to arise from the correct mode of action and, in any case, poor cellular activity is not unexpected for newly identified hits. Most important of all, this discovery validates docking-based virtual screening as a viable strategy for hit identification against Hsp90.

Crystallographic coordinates have been deposited with the Protein Data Bank: PDB code 2BZ5.

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