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Fragment-based Identification of Hsp90 Inhibitors

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Heat shock protein 90 (Hsp90) is an ATP-dependent molecular chaperone involved in the conformational maturation of numerous client proteins implicated in diverse cellular functions.[1] Moreover, Hsp90 plays a key role in the stress response and protection of the cell against the effects of mutation.[2] Many of the Hsp90 client proteins are overexpressed and/or mutated in cancer (for example, ERBB2, CDK4, C-Raf, B-Raf, c-Met, h-Tert), and are directly associated with cancer progression.[3] The inhibition of Hsp90 and subsequent loss of chaperone function causes client proteins to be degraded by the ubiquitin proteasome pathway. Hsp90 inhibitors therefore offer a concerted attack on the unrestricted proliferation and survival of cancer cells, leading to cell growth inhibition and apoptosis.

Hsp90 protein consists of three distinct domains[4] two of which have been the focus of drug discovery efforts. The C-terminal domain has been shown to interact with novobocin and cisplatin, and it appears to have a role in the homodimerisation process.[5] The middle domain is involved in the ATPase cycle and in the binding with co-chaperone and client proteins.[6] Finally, the N-terminal ATP binding domain interacts with natural product inhibitors (for example, geldanamycin and radicicol) and a number of newly identified chemical entities.[7] This domain is a tractable target which is amenable to structural biology approaches that facilitate structure-based inhibitor optimisation.[8]

Validation of Hsp90 as a protein target for drug discovery comes from the results of clinical studies with 17-allylamino-17-demethoxygeldanamycin (17-AAG)[9] or its closely related "soluble" analogue 17-dimethylaminoethylgeldanamycin (17-DMAG)[10] and the prodrug of 17-AAG, IPI-504.[11] However, the inherent chemical complexity of the benzoquinone ansamycin scaffold combined with limited solubility, hepatotoxicity, and extended metabolism,[12] has led to significant efforts directed towards the identification of novel small-molecule inhibitors of Hsp90. Indeed a number of novel small-molecule inhibitors are currently in clinical trials, for example, SNX-5422[13] and NVP-AUY922.[14]

Fragment-based screening has rapidly become a proven technique to identify novel chemical starting points in drug discovery programs. Furthermore, the rapid optimisation of fragment hits using structure-based design has established fragment-based drug discovery (FBDD) as a valuable strategy in the search for new drug molecules. Herein, we describe the application of fragment-based drug discovery to the identification of potent ligands for the N-terminal ATP binding domain of Hsp90.

Our approach to fragment screening for Hsp90 was to use a high concentration confocal fluorescence-based biochemical assay whereby fragments were identified that displaced a Tamra-labelled analogue of geldanamycin.[15] A proprietary library of 20 000 fragments was screened, and multiple fragments that bound were identified. Promising hits were submitted to co-crystallisation and soaking experiments with the N-terminal domain of Hsp90.

Analysis of multiple crystal structures of diverse fragment complexes of Hsp90 derived from the primary fragment screen made evident the flexibility of Hsp90 and key conserved interactions (manuscript in preparation). In particular Hsp90 was found to adopt a helical conformation in the region of Asn105 to Ile110 in the presence of a subset of the fragments. The helical conformation of Hsp90 creates a compact and well defined pocket adjacent to the adenosine binding site. Furthermore, a significant number of the fragment hits were found to contain the aminopyrimidine substructure, this motif has been consistently found to bind in the ATP binding site of Hsp90.

Fragment **1** (IC_{50} (Hsp90): 15 μM), presented a novel nonplanar bicyclic arrangement and was selected for further optimisation. In the crystal structure of the complex with **1** (Figure 1), the key interaction with the protein was found to be with the 2-amino group and the Asp93 residue, located in the ATP binding site. Furthermore a network of hydrogen bonds with conserved water molecules and the fragment were also observed. In this structure the entrance to the helical pocket was closed. However, structure-guided modifications of compound **1** were

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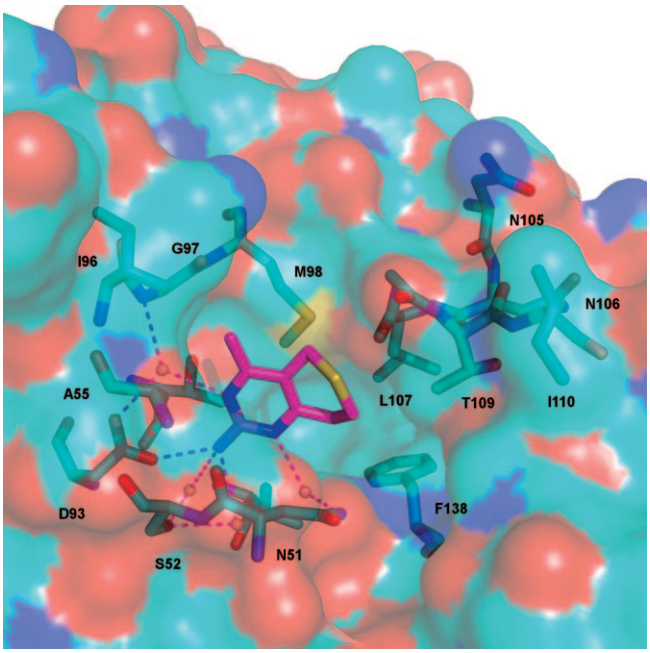


Figure 1. Crystal structure of the complex of **1** and Hsp90 (PDB code: 3FT5).

designed to reach this pocket and to exploit favourable interactions within this region of the active site.

Substructure searches of fragment **1** were performed against an in silico library of 3.8 million commercially available screening compounds. The hits from these searches were docked into two crystal structures of Hsp90, one with the helical pocket open and one with the helical pocket closed. Docking was performed with GOLD v 3.0.1,[16] using the GOLD scoring function. Docked poses were inspected visually to identify compounds that both maintained the key interactions between the aminopyrimidine, Asp93, and the conserved waters and accessed the helical pocket, or provided an appropriate vector to access the helical pocket. A small set of compounds was selected and purchased for biochemical evaluation. This led to the discovery of tetrahydrobenzopyrimidines **2** and **3**, which, in addition to showing a modest improvement in potency against Hsp90, were also considered more amenable for further evolution (Figure 2).

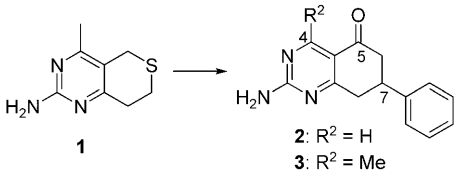
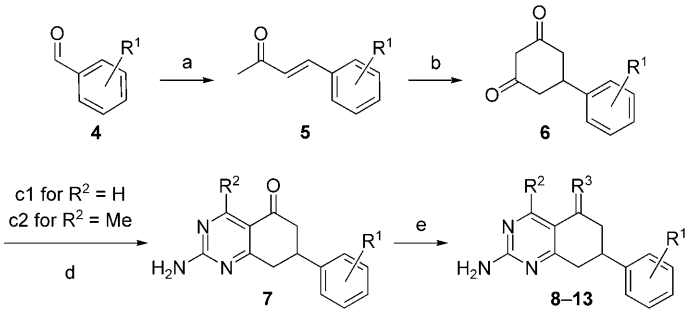


Figure 2. Initial fragment hit and fragment evolution starting point.

Following a structure-guided approach, further analogues were prepared focusing on the exploration of the substitution of the phenyl ring and also of the 4- and 5-positions. Modified literature conditions[17] were employed to prepare these compounds. Their synthesis (Scheme 1), involved the preparation of diversely substituted 3-phenylcyclohexan-1,3-diones **6**. These were then converted into their 2-aminopyrimidine scaffolds[18] and the 5-ketone position could be further functionalised to oxime derivatives.



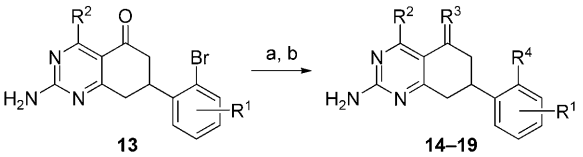
Scheme 1. Reagents and conditions: a) acetone, NaOH (aq), 2 h at 65 °C, 96%; b) $CH_2(CO_2Et)_2$, NaOEt, EtOH, 1 h at 65 °C, then 2 M NaOH (aq), 1 h at 80 °C, then conc. HCl (aq), 1 h at 100–120 °C, 72% over three steps; c) 1 DMF–DMA, $CHCl_3$, RT, 30 min, 81%; 2 AcCl, DCE, Et_3N, 2 h at RT, 66%, then KCN, Et_3N, MeCN, 16 h, 88%, then pyrrolidine, MS (4 Å), $CHCl_3$, 1 h at RT, 100%; d) guanidine carbonate, 1,4-dioxane, 16 h at 100 °C, 94%; e) $NH_2OH{\cdot}HCl$, pyridine, $CHCl_3$, 16 h at 60 °C, 63%

A selection of initial structure–activity relationships (SAR) is presented in Table 1. Introduction of a 4-methyl group (R^2) was in most cases beneficial to activity. This was partly attributed to preferential formation of the more active antioxime isomer because of the resulting steric hindrance. Compounds bearing an oxime in the 5-position (R^3) also showed enhanced activity relative to their ketone counterparts. Furthermore, the presence of a fluorine substituent in the *para* position of the phenyl group (R^1) was beneficial. At this stage, however, these initial compounds displayed negligible cellular activity.

Table 1. Activity of monoaryl inhibitors.

Compd	R^1	R^2	R^3	IC_{50} [μM] Hsp90
2	H	H	O	8.2
3	H	Me	O	0.8
8	H	H	NOH	1.3
9	4-F	H	O	4
10	4-F	Me	O	0.8
11	4-F	H	NOH	1.1
12	4-F	Me	NOH	0.5[19]

Following modelling studies with this first round of compounds it was suggested that the phenyl group in the 7-position was triggering the opening of the helical pocket. It was subsequently postulated that the introduction of an additional aryl moiety (R^4) at the *ortho* position of the first phenyl ring could generate a potential π-stacking interaction with Phe138 of Hsp90. The additionally substituted phenyl analogues were prepared (Scheme 2) using a Suzuki reaction to generate biaryl



Scheme 2. Reagents and conditions: a) $R^4B(OH)_2$, $[Pd(PPh_3)_4]$, K_2CO_3, toluene, EtOH, μ-wave (250 W), 30 min at 150 °C, 66–70%; b) $NH_2OH{\cdot}HCl$, pyridine, 3 h at 60 °C, 30–63%.

2-aminopyrimidines. The intermediate ketone derivatives were then further functionalised to the corresponding oximes.

Our hypothesis was confirmed, and on addition of a second phenyl ring, cellular activity was observed (Table 2). Investigation of the R^4 substitution rapidly revealed that the 4-pyridyl group was detrimental whereas the introduction of a 3-pyridyl group maintained similar biochemical and cellular levels. Submicromolar cellular activity was obtained with the 4-fluoro-3-pyridyl system **19**, on two different human cancer cell lines, A549 (non-small-cell lung cancer) and HCT116 (colon carcinoma).

Table 2. Activity of biaryl oxime inhibitors.

Compd	R^1	R^2	R^4	IC_{50} [μM] Hsp90	A549	HCT116
14	H	H	Ph	0.40	5.7	6.39
15	H	Me	Ph	0.057	4.20	8.10
16	H	Me	4-Py	1.1	ND[a]	ND[a]
17	H	Me	3-Py	0.053	4.25	4.19
18	4-F	Me	Ph	0.27	1.77	1.62
19	4-F	Me	3-Py	0.03	0.85	0.85

[a] Not determined.

The analysis of the molecular signature of Hsp90 inhibition on cells, comprising the induction of Hsp70 and the depletion of a well-known Hsp90 client protein such as C-Raf was carried out. The data reported in Figure 3, confirmed the ability of the molecule to inhibit the target on cells in a concentration dependent manner in both cell lines as determined by western blotting.

The co-crystal structure of analogue **19** with Hsp90 was elucidated and confirmed the predicted interactions (Figure 4).

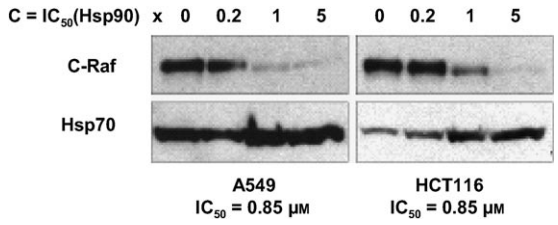


Figure 3. Western Blot of compound **19** on A549 and HCT116 cell lines. Target modulation at 24 h tested at 0, 0.2, 1, and 5 times the respective cellular IC_{50} value.

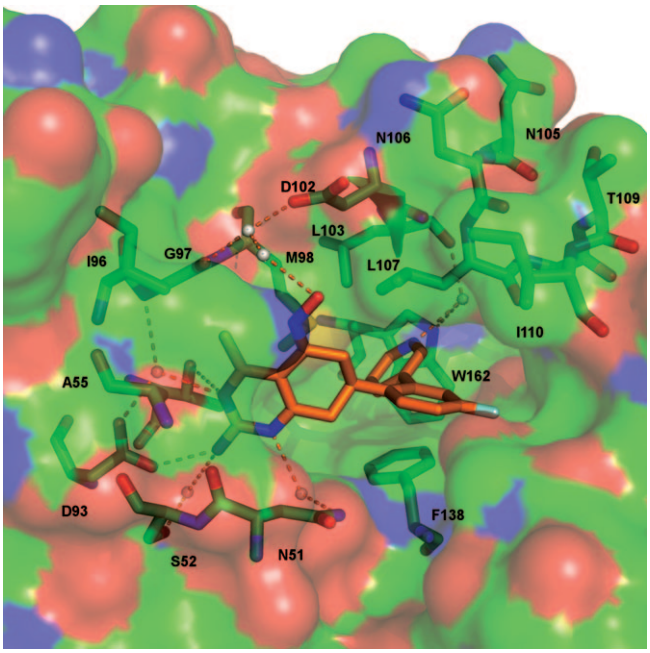


Figure 4. Crystal structure of complex of **19** and Hsp90 (PDB code: 3FT8).

The pyridyl ring not only undergoes π-stacking interactions with Phe138 but is also involved in a hydrogen bond interaction with a water molecule located in the helical pocket. Other noteworthy interactions are observed with the oxime moiety that is involved in a network of interactions with water molecules.

In summary, through a high-throughput biochemical fragment screen we have identified novel small molecules that are potent Hsp90 inhibitors. The fragment hits were rapidly optimised using a combination of in silico commercial analogue selection and structure-based design. Further optimisation is ongoing and will be reported in a subsequent publication.

Keywords: fragment-based screening · Hsp90 · oximes · structure-based drug design · tetrahydrobenzopyrimidine

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[18] The preparation of analogues in which R^2 = methyl requires rearrangement of the *O*-acetylenone into a *C*-acetyl intermediate. The rearrangement was performed using a catalytic amount of KCN. Extreme care should be taken, as cyanide gas is produced during the workup. Adequate protective clothing and scrubbing should be used. Alternative thermal- or 4-*N*,*N*-dimethylaminopyridine-promoted rearrangement yielded the desired product but resulted in lower yields in our hands.
[19] 46 and 75% cell growth inhibition was observed respectively for A549 and HCT116 cell lines at 50 μM; IC_{50} values were not determined.

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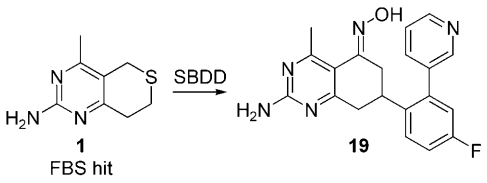

Heat shock protein 90 (Hsp90) plays a key role in stress response and protection of the cell against the effects of mutation. Herein we report the identification of an Hsp90 inhibitor identified by fragment screening using a high-concentration biochemical assay, as well as its optimisation by in silico searching coupled with a structure-based drug design (SBDD) approach.

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