FS23 binds to the N-terminal domain of human Hsp90: A novel small inhibitor for Hsp90 (Post-print)

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Abstract

The N-terminal domain of heat shock protein 90 (Hsp90N) is responsible for the catalytic activity of Hsp90. The reported inhibitors of Hsp90 bind to this domain and would inhibit tumor growth and progression. Here, we synthesized FS23, a small molecule inhibitor of Hsp90, and collected X-ray diffraction data of the complex crystal of Hsp90-FS23. High resolution X-ray crystallography shows that FS23 interacted with Hsp90N at the nucleotide binding cleft, and this suggests that FS23 may compete with nucleotides to bind to Hsp90N. The crystal structure and the interaction between Hsp90N and FS23 suggest a rational basis for the design of novel antitumor drugs.

Full Text

Preamble

FS23 binds to the N-terminal domain of human Hsp90: A novel small inhibitor for Hsp90

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The N-terminal domain of heat shock protein 90 (Hsp90N) is responsible for the catalytic activity of Hsp90. Reported inhibitors of Hsp90 bind to this domain and inhibit tumor growth and progression. Here, we synthesized FS23, a small molecule inhibitor of Hsp90, and collected X-ray diffraction data for the Hsp90-FS23 complex crystal. High-resolution X-ray crystallography shows that FS23 interacts with Hsp90N at the nucleotide binding cleft, suggesting that FS23 may compete with nucleotides for binding to Hsp90N. The crystal structure and the interaction between Hsp90N and FS23 provide a rational basis for the design of novel antitumor drugs.

Keywords: Heat shock protein 90, N-terminal domain, Inhibitor, X-ray diffraction, Interactions
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Introduction

X-ray crystallography is now used routinely to determine how a pharmaceutical interacts with its protein target and what changes may improve it. It offers detailed insights into protein-ligand interactions and holds promise for developing novel, more effective, safer, and cheaper drugs. During the past decade, macromolecular crystallography has become a standard technique employed by pharmaceutical and biotechnology industries.

Heat shock protein 90 (Hsp90) is a homodimer, with each monomer consisting of three domains: an N-terminal domain, a middle domain with a large hydrophobic surface that assists in folding client proteins, and a C-terminal domain that plays a prominent role in constitutive dimerization. The N-terminal domain (Hsp90N) contains a highly conserved ATP binding site that is essential for Hsp90 function. The first X-ray structure of human Hsp90N, reported by Stebbins et al., reveals an α/β sandwich with a pronounced ligand-binding pocket approximately 15 Å deep. The "floor" of this structure consists of eight uninterrupted antiparallel β sheets, while the pocket walls are formed by three α helices and a loop. A helical face of the sandwich features a surface groove that leads into the pocket.

Hsp90 serves dual chaperone functions: participating in the conformational maturation and activation of a wide range of client proteins, many of which play important roles in controlling proliferation, survival, invasion, metastasis, and angiogenesis; and mediating ATP-dependent refolding. These functions suggest that Hsp90 inhibitors may cause potent inhibition of tumor growth and progression. Hsp90 has attracted considerable interest as a therapeutic target for anticancer drugs since the discovery that both geldanamycin (GDM) and radicicol could inhibit Hsp90 function by binding to an ATP binding pocket in the N-terminal domain.

As a naturally occurring antitumor antibiotic, GDM was in preclinical development as an antitumor agent, but its mechanism was unknown until the crystal structure of the Hsp90N-GDM complex was determined, suggesting that GDM acts by blocking ATP binding to Hsp90. Once the inhibitor binds to the pocket, the conformational/domain rearrangement of Hsp90 no longer occurs, rendering Hsp90 unable to assist its client proteins in achieving conformational maturation, stability, and function. Naturally occurring antibiotics for Hsp90, such as radicicol and others, function similarly. Unfortunately, these antitumor antibiotics have side effects, including liver toxicity, prompting studies to find new approaches for cancer management through Hsp90α. Several derivatives of natural inhibitors, including 17-AAG, 17-DMAG, and IPI-504, have entered clinical studies. However, potential limitations include poor solubility, limited bioavailability, and hepatotoxicity, leading to significant efforts to identify small-molecule inhibitors.

Structure-based drug design (SBDD) has become a proven technique for finding novel compounds as starting points for optimization. Compounds are selected by docking and tested in biochemical assays. Confirmed hits are crystallized, and information from X-ray structures of the inhibitors is used to drive the drug evolution process.

To continue efforts to discover new Hsp90 inhibitors, we synthesized FS23, a new inhibitor designed by merging bioactive fragments identified from fragment compounds (selected by docking and tested in biochemical assays) through screening and optimization using the SBDD method. Subsequently, we resolved the crystal structure of the Hsp90N-FS23 complex by X-ray diffraction. The complex structure provides the structural basis for further investigation of the activated conformational changes in Hsp90 upon FS23 binding. The most interesting feature of the interface in the Hsp90N-FS23 complex is a hole produced by structural rearrangements of L6. There is neither direct nor indirect interaction between residues in the hole and FS23, suggesting that groups could be added to FS23 to fill this cavity and interact with residues in the hole to increase the activity of derivatives.

II. Experimental

A. Synthesis of the small molecule FS23

As shown in [Figure 1: see original paper], under reaction with lithium hexamethyldisilazide, Compound 1 was condensed with 4-methoxy phenylacetonitrile to give Compound 2, followed by cyclization with hydroxylamine hydrochloride to obtain Compound 3. This reacted with cyclopropanecarbonyl chloride, and subsequently underwent Suzuki reaction with isoquinoline-4-boronic acid to give Compound 5, which was deprotected by boron trichloride to yield the target compound FS23.

B. Protein purification and crystallization

The vector containing the gene for human Hsp90α N-terminal domain (residues 9-236) was cloned into a pET28a vector, and the recombinant plasmid was transformed into *E. coli* Rosetta (DE3) pLysS for over-expression (Invitrogen, Carlsbad, USA). Bacteria were grown in 800 mL of LB (Luria-Bertani) broth at 37 °C to an OD_{600} of 0.6–0.8, then induced with 200 µM IPTG (isopropyl β-D-thiogalactopyranoside) for 3–5 h at 30 °C.

Bacteria were collected by centrifugation at 10,000 g for 10 min (CF16RX, Hitachi). The precipitate was re-suspended in buffer A (100 mM Tris/HCl buffer, pH 7.5, 300 mM NaCl, and 5% glycerol) and lysed using a JNBIO 3000 plus (JNBI). The lysate was centrifuged at 30,000 g for 30 min at 4 °C, and the supernatant was loaded onto a 5 mL Ni-NTA (Ni^{2+}-nitrilotriacetate) column (GE Healthcare). Protein was eluted with buffer B (100 mM Tris-HCl buffer, pH 7.5, 300 mM NaCl, 100 mM imidazole, and 5% glycerol). The protein product was concentrated and buffer-exchanged to buffer C (100 mM Tris-HCl buffer, pH 7.5, 300 mM NaCl, and 5% glycerol) using an Amicon Ultra-15, 10,000 Mr cut-off centrifugal concentrator (Millipore). For further purification, impurities were removed by a Superdex 75 PG gel-filtration column (GE Healthcare) with buffer C. Positive fractions were analyzed by 15% SDS-PAGE to determine purity. The protein was flash-frozen in liquid nitrogen and stored at -80 °C.

For crystallization, the purified Hsp90 N-terminal domain was concentrated using an Amicon Ultra-15, 10,000 Mr cut-off centrifugal concentrator to approximately 20 mg/mL. The small molecule FS23 was added to the protein at a 5:1 molar ratio, and the mixture was incubated for 1 h at 4 °C. After incubation, the mixture was centrifuged for 10 min and precipitate was removed. The supernatant was analyzed by DLS (dynamic light scattering, Malvern) to determine whether the complex was suitable for crystallization, following the standard protocols supplied by the manufacturer. Crystallization was carried out at 4 °C using the hanging drop vapor diffusion method. Cocrystals were grown under conditions similar to those previously described for native crystals, with slight modifications: 100 mM Tris-HCl, pH 8.5, 20% PEG 4000, 200 mM $MgCl_2$.

C. Data collection, structure determination, and refinement

Crystals were mounted and flash-frozen in liquid nitrogen for diffraction testing and data collection. All data sets were collected at 100 K on beamline BL17U1 at the Shanghai Synchrotron Radiation Facility (SSRF) and processed with the HKL2000 software package. Structures were solved by molecular replacement using PHENIX. The search model was the previously reported structure of Hsp90N (PDB code 3T0H). Structures were refined using PHENIX. With the aid of the program Coot, water molecules and other features were fitted into the initial Fo-Fc map. Complete statistics and quality metrics for the solved structure are shown in .

III. Results and Discussion

A. Purification of Hsp90N

Hsp90N was purified to apparent homogeneity using metal-chelating chromatography followed by gel filtration chromatography. The elution volume peak corresponded to monomeric Hsp90N with an apparent molecular weight of 25 kDa and showed good purity (assessed by SDS-PAGE to be 98%), as shown in Figure 2: see original paper. Protein and FS23 were mixed at a 1:5 molar ratio, and DLS results showed only one peak following Gaussian distribution, indicating uniform particle diameter. The PDI (Polydispersity Index) was 13.7%, suggesting that nearly all the complex existed as monomers, as shown in Figure 2: see original paper. These data demonstrated that the mixture was suitable for crystallization.

B. Crystallization and structure determination

After 3-4 days, crystals grew with average dimensions of approximately 200 µm × 100 µm × 50 µm ([Figure 3: see original paper]). Diffraction data were collected to ~1.70 Å resolution ([Figure 4: see original paper]) and indexed in space group I222. The unit-cell parameters were a = 70.06 Å, b = 88.27 Å, c = 97.00 Å; $\alpha = 90°$, $\beta = 90°$, and $\gamma = 90°$.

The Hsp90N-FS23 structure was solved by molecular replacement using the known 3-D structure of Hsp90N as a search model (PDB code 3T0H) and determined at 1.70 Å resolution (PDB code 5CF0). In the crystal structure, FS23 binds completely to the ATP-binding pocket, and its intact electron density has been captured, as shown in Figure 5: see original paper. We therefore have reason to believe that FS23 was fully bound in the Hsp90N complex structure.

The refined model contains residues Val17-Lys224, and no electron density was observed for residues Asp9-Glu16 and Glu225-Glu236. These missing residues are at the N- and C-termini and are presumed to be disordered.

C. Analysis of crystal structure of Hsp90N-FS23 complexes

Although the refined structure of Hsp90N-FS23 differs from that of Hsp90N (PDB code 3T0H) in resolution, no significant conformational changes were observed between the two protein structures, as shown in Figure 6: see original paper. However, there are minor differences from Asp102 to Tyr139 between the two structures. The Hsp90N-FS23 complex structure provides the structural basis for further investigation of the activated conformational change of Hsp90 upon FS23 binding. Superimposition of the Hsp90N-FS23 complex with native Hsp90N structures shows that helices H4 (amino acids from Thr99 to Leu107) and H5 (amino acids from Gly114 to Ala124) of Hsp90N undergo conformational rearrangement in response to bound FS23 (Figure 6: see original paper).

The crystal packing patterns of Hsp90N-FS23 (I222) and Hsp90N-ATP ($P2_1$) differ, and the region from Met98 to Tyr139 also differs between the two struc-

tures (Figure 6: see original paper). In the Hsp90N-ATP complex, a loop containing amino acids from Leu107 to Thr115 is called L6 (Figure 6: see original paper). The ATP lid, consisting of H4-L6-H5-H6 secondary structure elements, adopts two different conformations in Hsp90N that are essential for regulating the entrance and width of the pocket.

In free Hsp90N, the lid is in an open conformation and the pocket is about 12 Å in diameter near its entrance. When ATP binds to the pocket, H4 and H5 undergo helix-to-coil and coil-to-helix transitions that drive L6 to move into the pocket, replacing H4 as one of the pocket walls. The ATP lid then changes to a closed conformation, with the functional consequence that the L6 loop acts as a gate to constrict the pocket entrance from 12 Å to 8 Å. Only when the ATP lid is in the open conformation can Hsp90 interact with its client proteins to accomplish conformational maturation and activation. However, in Hsp90N-FS23 there is only a large helix from Thr99 to Ala124, meaning the L6 loop has been replaced by a helix. This indicates that even when substrate binds to Hsp90, conformational change cannot occur, resulting in failure to interact with client proteins (Figure 6: see original paper). The disappearance of L6 in the Hsp90N-FS23 complex plays an important role in explaining why FS23 can block Hsp90 function.

Electrostatic potential surfaces surrounding the active sites of Hsp90N-FS23 and Hsp90N-ATP are shown in [Figure 7: see original paper]. Like the Hsp90N-ATP structure, the FS23 binding site is located in the ATP binding site and adopts the shape of a cleft (Figure 7: see original paper). Interestingly, inhibitor binding induces structural rearrangements of L6 and produces a hole in the structure (Figure 7: see original paper).

Interactions between Hsp90N and FS23

There are two direct hydrogen bonds between Hsp90N and FS23. One is the interaction between FS23 and Asp93 at a distance of 2.7 Å. The other is the interaction between FS23 and Thr184 at a distance of 3.5 Å. π-π stacking interactions are formed between the side chain of residue Phe138 and the aromatic rings of FS23. FS23 forms water-mediated hydrogen bonds with residues Leu48, Ser52, Ile96, Asp102, and Gly135. This hydrogen bond network is the most critical portion of the interface and enables FS23 to bind Hsp90N effectively ([Figure 8: see original paper]).

Furthermore, there is extensive, though not complete, surface complementarity between FS23 and the pocket, allowing for a high density of van der Waals contacts. Superimposition of Hsp90N-FS23 (PDB code 5CF0) with Hsp90N-ATP (PDB code 3T0Z) and Hsp90N-GDM (PDB code 1YET) structures reveals that FS23, ATP, and GDM occupy the same location, though with subtle differences in chemical groups. In the Hsp90N-GDM complex (PDB code 1YET), for example, geldanamycin nearly occupies the entire pocket, leaving little room for modification, making it difficult to achieve significant improvements in solubility, bioavailability, and hepatotoxicity in geldanamycin derivatives. The situation

is different for FS23 binding, where there is sufficient room for redesign and modification to obtain derivatives with better properties ([Figure 9: see original paper]).

The pocket is mostly polar at its entrance, so hydrophilic groups that can interact with polar amino acids could be added to FS23 to increase the solubility of the small molecule. The pocket becomes predominantly hydrophobic near the bottom, and derivatives with increased affinity for Hsp90 may be obtained if groups with non-hydrogen atoms are added to FS23 and hydrogen bond complementarity is increased. The most interesting feature of the interface in the Hsp90N-FS23 complex is the hole produced by structural rearrangements of L6. There is neither direct nor indirect interaction between residues in the hole and FS23, so groups can be added to FS23 to fill this cavity and interact with residues in the hole to increase the activity of derivatives.

IV. Conclusion

The structure of Hsp90N reveals a pronounced, highly conserved pocket, and residues in the pocket are confirmed to be essential for maintaining protein activity. Compound FS23 was designed to interact with these residues and has been identified as a potential Hsp90 inhibitor. In the crystal structure, intact electron density of FS23 has been captured, leading us to believe that FS23 was fully bound in the Hsp90N complex structure.

There are differences from Met98 to Tyr139 between the Hsp90N-FS23 and Hsp90N-ATP structures. The disappearance of L6 in the Hsp90N-FS23 complex plays an important role in explaining how FS23 blocks Hsp90 function. Interestingly, inhibitor binding induces structural rearrangements of L6 and produces a hole in the structure.

Superimposition of Hsp90N-FS23 with Hsp90N-GDM structures reveals that FS23 and GDM occupy the same location but with subtle differences in chemical groups. There is sufficient room for redesign and modification of FS23 to obtain derivatives with better properties. Hydrophilic groups that can interact with polar amino acids could be added to FS23 to increase solubility, and groups could be added to fill the cavity produced by structural rearrangements of L6 and interact with residues in the hole to increase derivative activity. Overall, there is considerable potential for FS23 derivatives with excellent characteristics, and the FS23 binding pattern to Hsp90N provides a new approach for designing protein inhibitors.

This new Hsp90 inhibitor FS23 explores a region by extending ligands into a less-explored sub-pocket and provides a potential chemotype for Hsp90 inhibitor development. The Hsp90N-FS23 structure offers detailed protein-inhibitor interactions and holds promise for novel, more effective, safer, and cheaper drugs.

Each year, new targets are identified, structures of those targets are determined at an amazing rate, and our capability to quantitatively capture interactions

between targets and ligands is accelerating. SBDD is a powerful method, especially when used as a tool within an armamentarium for discovering new drug leads against important targets. After a target and its structure are chosen, new leads can be designed from chemical principles to create very promising candidates that can advance to Phase I clinical trials.

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Note: Figure translations are in progress. See original paper for figures.