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A fluorescence polarization assay for inhibitors of Hsp90

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Abstract

Hsp90 encodes a ubiquitous molecular chaperone protein conserved among species which acts on multiple substrates, many of which are important cell-signaling proteins. Inhibition of Hsp90 function has been promoted as a mechanism to degrade client proteins involved in tumorigenesis and disease progression. Several assays to monitor inhibition of Hsp90 function currently exist but are limited in their use for a drug discovery campaign. Using data from the crystal structure of an initial hit compound, we have developed a fluorescence polarization assay to monitor binding of compounds to the ATP-binding site of Hsp90. This assay is very robust ($Z' > 0.9$) and can detect affinity of compounds with IC_{50}s to 40 nM. We have used this assay in conjunction with cocrystal structures of small molecules to drive a structure-based design program aimed at the discovery and optimization of a novel class of potent Hsp90 inhibitors.

Keywords: Hsp90; Fluorescence polarization; ATPase; SBDD; SAR; Resorcinol; Geldanamycin; 17AAG; Radicicol; PU3

The 90-kDa heat-shock protein, Hsp90, is a highly conserved molecular chaperone protein (for reviews on Hsp90 see [1–5]). It is highly abundant in the cell and has been shown to be essential for cell survival. There are four forms present in humans; Hsp90α (inducible upon stress), Hsp90β (low-level constitutive expression), Grp94 (endopolasmic reticulum localized), and TRAP1/Hsp75 (mitochondrial matrix localized). Hsp90 functions as a dimer, both homo- and heterodimers, to maintain the appropriate folding and conformation of many other proteins [6]. The ATPase activity of Hsp90 is located in its N-terminal domain [7,8]. However, the basal ATPase activity of Hsp90 is low and it requires two associate proteins, Aha1 and Hch1, to achieve full activity precluding direct assaying of inhibition of this activity [9–12]. Hsp90 is unique among chaperone proteins in that its partner proteins are involved in many cellular pathways critical for cell growth and survival. Hsp90 regulates many important kinases, for example erbB2 and Raf1, and other key cellular proteins [13–15].

In many tumors Hsp90 expression is amplified. The accumulation of Hsp90 aids survival of the tumor cell by refolding partially damaged or mutant proteins and stabilizing them, for example mutant p53 [8,16]. Many of the signaling proteins associated with Hsp90 are serine/threonine kinases which have essential roles in malignant transformation. For example Raf1, a key member of the Ras-MAPK signaling pathway, has been shown to exist in a complex with Hsp90 and this binding is essential for the activity of Raf1 [13].

Inhibition of Hsp90 activity has been shown to selectively degrade many of the client proteins involved in cell proliferation, cell cycle regulation, and apoptosis in several tumor models [5]. The result of degradation of these proteins is either cell stasis or apoptosis of the tumors [17].

There are several small-molecule Hsp90 inhibitors currently reported in the literature. The ansamycin, geldanamycin, was the first Hsp90 inhibitor identified and has been

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shown to bind to the ATP-binding pocket in the N-terminal domain [18]. Geldanamycin has antitumor effects but progress through clinical trials has been halted due to off-target toxicity [16,19]. Several derivatives of geldanamycin have since been developed (17-AAG and 17-DMAG) and these are currently in clinical trials [20,21]. The antibiotic radicicol inhibits Hsp90 activity in vitro but has failed to show tumor suppression in in vivo models due to instability of the compound [22,23]. However, oxime derivatives of radicicol show in vivo activity and it is expected that these will be progressed to the clinic [24]. PU3, a purine derivative, binds to the ATP-binding pocket of Hsp90 and has been shown to prevent tumor growth in vitro [25–27].

To detect Hsp90 inhibitors and generate SAR,[1] we have developed a high-throughput assay to monitor inhibition of Hsp90 activity. Many assays have been described to monitor inhibition of Hsp90 [53,54] using several different techniques, e.g., time-resolved fluorescence resonance energy transfer and fluorescence polarization (FP). However all these assays use derivatives of natural products which have been shown to inhibit Hsp90 function. Due to the limited sites of modification available on natural products we decided to develop an assay using a derivatized small molecule. Due to the availability of a crystal structure of a lead compound in Hsp90 we were able to rationally design a probe for a fluorescence polarization assay. The assay is based upon displacement of a fluorescently labeled molecule, which binds specifically to the ATP-binding site of full-length human Hsp90. We monitor this displacement by a decrease in fluorescence polarization of the probe-Hsp90 complex when the inhibitor binds. The assay is very robust ($Z' > 0.9$) and can detect inhibitor binding to IC_{50}s of 40 nM. The assay has been developed to identify inhibitors of both full-length human Hsp90α and Hsp90β. We have used this assay to screen known Hsp90 inhibitors and have compared this to inhibition of the ATPase activity of yeast Hsp90, an assay previously used to monitor Hsp90 inhibition [28,29].

Materials and methods

Expression vector construction

pRSETA Human Hsp90β was obtained from C. Podromou (ICR, UK). pET19-scHsp90, encoding N-terminally His-tagged yeast Hsp90, was made in the following way. A 2.1-kb fragment encoding Hsp90 was amplified by PCR using Hotstart Pfu Turbo DNA Polymerase (Stratagene) according to the manufacturer's instructions from genomic DNA of *Saccharomyces cerevisiae* strain L40coat (Invitrogen). Primer sequences were Forward (cgc gca tat ggc tag tga aac ttt tga att tc) and Reverse (cgc gct cga gtt act aat cta cct ctt cca ttt cgg). This fragment was subcloned in pCR2.1 using TopoTA cloning kit (Invitrogen) to create the plasmid pCR-scHsp90. The insert was fully sequenced to confirm the correct open reading frame. A 2.1-kb *Nde*I-*Xho*I fragment was subcloned into pET19b (Novagen) to produce the plasmid pET19-Hsp90.

pET19-Hsp90α(9-236), encoding the His-tagged N-terminal ATPase domain of human Hsp90a, was constructed as follows. The region of Hsp90α encoding amino acids 9–236 was amplified by PCR from IMAGE clone 4026275 using primers Hsp90-N-HisFor (cgc ata tgg acc aac cga tgg agg ag) and Hsp90-N-Rev (gcg gat cct cat tat tca gcc tca tca tcg ct) using Hotstart Pfu Turbo DNA polymerase (Stratagene) and subcloned into pCRII using TopoBlunt cloning kit (Invitrogen) to create plasmid pCR-Hsp90α(9-236). The insert was fully sequenced to confirm that the expected region of Hsp90α was present. A 0.7 kb *Nde*I-*Bam*HI fragment from this plasmid was subcloned into pET19b (Novagen) to create the plasmid pET19-Hsp90α(9-236).

Hsp90 protein expression and purification

Expression and purification of full-length Hsp90β and His-tagged N-terminal Hsp90α was as previously described [30]. Yeast Hsp90 was overexpressed in the *Escherichia coli* strain BL21 and purified in a manner similar to that of His-tagged N-terminal Hsp90α.

Crystallization and three-dimensional structure determination

Apo Hsp90α protein was concentrated to approximately 20 mg/ml using ultrafiltration into a final buffer containing 20 mM Tris, pH 7.4, and 0.5 M NaCl. For cocrystallization experiments, a 20 mM stock solution of the ligand VER-00063579 in 100% DMSO was provided. At this concentration the ligand is at a twofold molar excess when added to the protein. The complex sample was left on ice for 1 h prior to setting up the crystallization trials. Cocrystals appeared overnight in conditions very similar to those for apo crystals, previously described [30]. These were subsequently transferred to cryoprotectant solution (crystallization reservoir solution with polyethylene glycol concentration increased from 25 to 35%) and frozen in liquid nitrogen, and data were collected on station ID29 at the European Synchrotron Radiation Facility (Grenoble, France).

Diffraction data were processed using DENZO [31]. The VER-00063579-bound structure was solved by molecular replacement with AMoRe [32] using the apo Hsp90α structure as the search model (PDB code [33] = 1UY1). All model building was carried out using the molecular graphics program O [34] and refinement calculations were performed with Refmac5 [35]. Following structure solution, difference electron density maps were calculated for the initial model, the ligand structure was modeled into the difference density peaks, and the coordinates were refined before addition of crystallographic water molecules using Refmac5 cycled with ARP/wARP [36]. The progress of the

[1] *Abbreviations used:* SAR, structure–activity relationships; FP, fluorescence polarization; DMSO, dimethyl sulfoxide; SEM, trimethylsilylethoxymethoxy.