Probe Report
March 13, 2008

Project Title: HTS for Tumor Hsp90 Inhibitors
Grant Number: 1 R03 MH076499-01

Screening Center Name: Emory Chemical Biology Discovery Center
Principal Investigator of Screening Center: Ray Dingledine

Assay Submitter & Institution: Gabriela Chiosis/Memorial Sloan-Kettering Cancer Center, New York

Assay or Pathway Target: Tumor Hsp90 protein

Probe PubChem Compound Identifier (CID): 245086

Assay provider information

Specific Aim: The long-term goal of this project is to identify novel tumor Hsp90 inhibitors with potential for use in cancer therapy and in the treatment of neurodegenerative diseases.

Significance: The biological role of the heat shock protein 90 (Hsp90) is mediated by its ability to interact with client substrates. Hsp90 is required for the ATP-dependent refolding of denatured or "unfolded" proteins and for the conformational maturation of a subset of proteins involved in the response of cells to extracellular signals. Activation of signaling pathways mediated by these Hsp90 clients is necessary for cell proliferation, regulation of cell cycle progression and apoptosis (Chiosis et al., 2004; Kamal et al., 2004; Maloney and Workman, 2002). Additionally, gain-of-function mutations responsible for transformation often require Hsp90 for maintenance of their folded, functionally active conformations.

Hsp90-interfering drugs represent a novel class of therapeutic agents that by selective inhibition of the chaperone could exhibit a broad-range of anti-tumor activity by affecting multiple aspects of transformation regulated by Hsp90. In addition, these agents represent a potential novel class of drugs that promote the survival of neurons and open up a promising approach for the treatment of neurodegenerative diseases (Solit and Chiosis, 2008).

Rationale: In order to fully explore the potential for Hsp90-targeted therapies, novel agents that interfere with the activity of the chaperone and have more drug-like properties are desirable. One method of identifying new classes of compounds active against the chaperone is to screen large libraries of compounds for Hsp90 binding. In transformed cells, Hsp90 is present in multi-chaperone complexes with high ATPase activity, whereas Hsp90 from normal tissues is in a latent, uncomplexed state. Therefore, it would be more therapeutically significant to measure affinity for Hsp90 in its cellular state and not the recombinant protein. Here, the Hsp90 binding FP assay was optimized by using cell lysate as the source of Hsp90 instead of recombinant Hsp90 protein. The assay measures binding to average Hsp90 population found in cell specific complex, thus with transformation-specific Hsp90. A red-shifted geldanamycin (cy3B-GM) was used in the Hsp90 FP assay to minimize interference from autofluorescent compounds (Du et al., 2007).

Screening center information:

Assay Implementation and Screening

- **PubChem Bioassay Name:** HTS for Tumor Hsp90 Inhibitors
- **List of PubChem bioassay identifiers generated for this screening project (AIDS):** 429; 712; 754
- **Primary Assay Description as defined in PubChem:** Hsp90 is a chaperone with important roles in maintaining transformation and in elevating the survival and growth potential of cancer cells. Recent evidence suggests additional applications of Hsp90 inhibitors in neurodegenerative diseases, nerve injuries, inflammation and infection. Several natural products that inactivate Hsp90 function have anti-tumor effects in *in vitro* and *in vivo* models of cancer. However, due to the role of Hsp90 in normal cellular homeostasis, it remained unclear whether Hsp90 inhibitors will be sufficiently specific for use as therapeutic agents. Early clinical results with 17AAG, the first Hsp90 inhibitor to enter clinical trials, confirm that Hsp90 is a promising target for novel cancer therapeutics and pave the road for the introduction of Hsp90 inhibitors in the clinic. The potential of Hsp90 inhibitors as therapeutics in other diseases has been less explored. Thus, there is increasing interest in developing novel inhibitors of this protein.

  A fluorescence polarization-based HTS assay has been developed and optimized for the identification of Hsp90 inhibitors by using tumor cell lysate Hsp90 (Du et al., 2007). Geldanamycin (GM) is a natural product that inhibits Hsp90 by binding to its ATP pocket (Neckers et al., 1999). The FP assay for the Hsp90 in cell lysate is based on the competitive binding of fluorescently (Cy3B) labeled geldanamycin.

  Data analysis:
  Percentage of inhibition is calculated by the equation based on per plate:

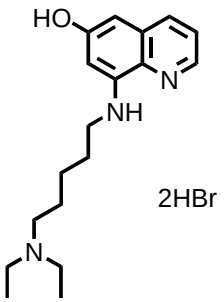
  % of Inhibition = 100 - ((mPc - mPf)/(mPb - mPf)) * 100

  Where mPc is the recorded mP from compound wells; mPf is average recorded mP from cy3B-GM only wells; mPb is average recorded mP from wells containing cy3B-GM and NCI-N417 lysate. An activity cutoff was set as a percent inhibition greater than 50%.

- **Center Summary of the Primary Screen:** 63918 compounds were screened in the primary HTS assay, and 223 hit compounds were identified. The plate Z' values are all above 0.6 and S/N above 10. The primary Hit rate is 0.35%.

Probe Optimization

- Chemical name of probe compound: 8-(5-diethylaminopentylamino)quinolin-6-ol dihydrobromide
- Draw probe chemical structure and show stereochemistry if known: see Figure 1
- Describe mode of action for biological activity of probe: This probe inhibits Hsp90 with a mean K_i of 101.8 nM in the Hsp90 binding FP assay and a mean IC_{50} of 1.0 μM in a cell-based Hsp90-client protein Her2 degradation assay.
- Has this compound been provided to the MLSMR?: Yes
- Description of secondary screens used to optimize probe structure:
  1. Dose-response Hsp90 binding FP assay: We tested the dose-response effect of hit compounds in Hsp90 binding FP assay to obtain the IC_{50} of the



Probe1

Fig. 1. Structure of Probe1. Chemical Name: 8-(5-diethylaminopentylamino) quinolin-6-ol dihydrobromide. Molecular Weight: 463.25g/mol; Molecular Formula: C18H29Br2N3O

compounds, and K_i values were calculated accordingly (Nikolovska-Coleska et al., 2004)

2. Cell-based Her-2 degradation assay in MCF-7 cells, a breast cancer cell line: Hsp90 uniquely stabilizes the Her2/Hsp90 association (Xu et al., 2001). Addition of Hsp90 inhibitors to cancer cells induces the proteasomal degradation of a small subset of proteins involved in signal transduction such as Raf1 kinase, Akt and certain transmembrane tyrosine kinases such as Her2. Thus, Her2 degradation in cells is a functional read-out of Hsp90 inhibition. Correlation between Hsp90 binding and Her2 degradation in cancer cells is indicative of a selective biological effect in these cells via Hsp90. We use a Western blot-based assay to measure the cellular level of Her2 protein in MCF-7 breast cancer cell lysates collected after 24 hours of compound treatment.

- Center comments on chemistry strategy leading to probe identification: The probe compound is commercially available and was purchased as an analog of an original hit compound identified in the primary screen. The activity of the commercial compound was confirmed by re-synthesis in house. The original hit compound (SID 850375) was identified in the primary HTS and was later shown to have a *Ki* = 333.0 nM in the Hsp90 binding FP dose response assay and IC_{50} = 5.0 μM in the cell-based Her2 degradation assay. The chemical strategy for optimizing SID 850375 involved modification of sections A, B, and C (Fig. 2). Structurally diverse 8-amino-6-alkoxy (or hydroxyl) derivatives in sections A and B were prepared by two different synthetic approaches (Scheme 1). In section A, various substituents with different carbon chain lengths were investigated, whereas several functional groups such as hydrogen, halogen, methoxy, benzyloxy, and hydroxyl were introduced in section B. To modify the quinoline ring in section C, halogens (Cl, Br) and substituted or unsubstituted amino groups were introduced. Based on these modifications, a total of 101 synthetic derivatives and 41 commercial compounds purchased from NCI were prepared for testing. Structure confirmation, re-synthesis, and SAR analysis led to the identification of several aminoquinoline analogs as nanomolar potency inhibitors of Hsp90 based on the initial hit structure (**Fig. 2**). The best compound in this series, namely probe**1,** exhibits a low-nanomolar binding affinity with a mean *Ki* = 101.8 nM in the Hsp90 binding FP assay. Probe**1** also shows low-micromolar activity (mean IC_{50} = 1.0 μM) in the cell-based Her2 degradation assay (Table 2). Compounds were classified into two subclasses, namely, 8-alkylaminoalkyl-6-methoxy-quinolines and 8-alkylaminoalkyl-6-hydroxyl-quinolines as shown in **Fig. 3**.

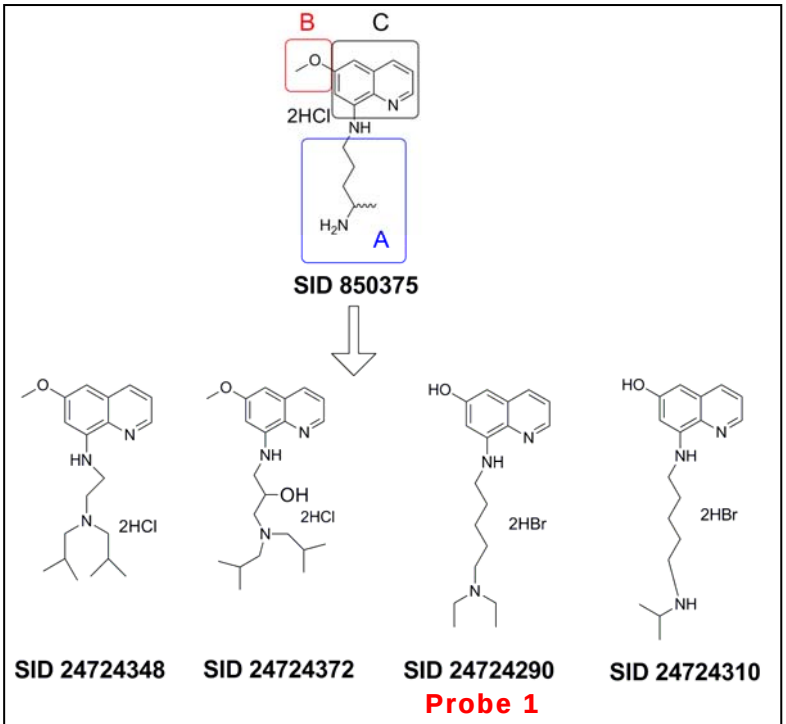
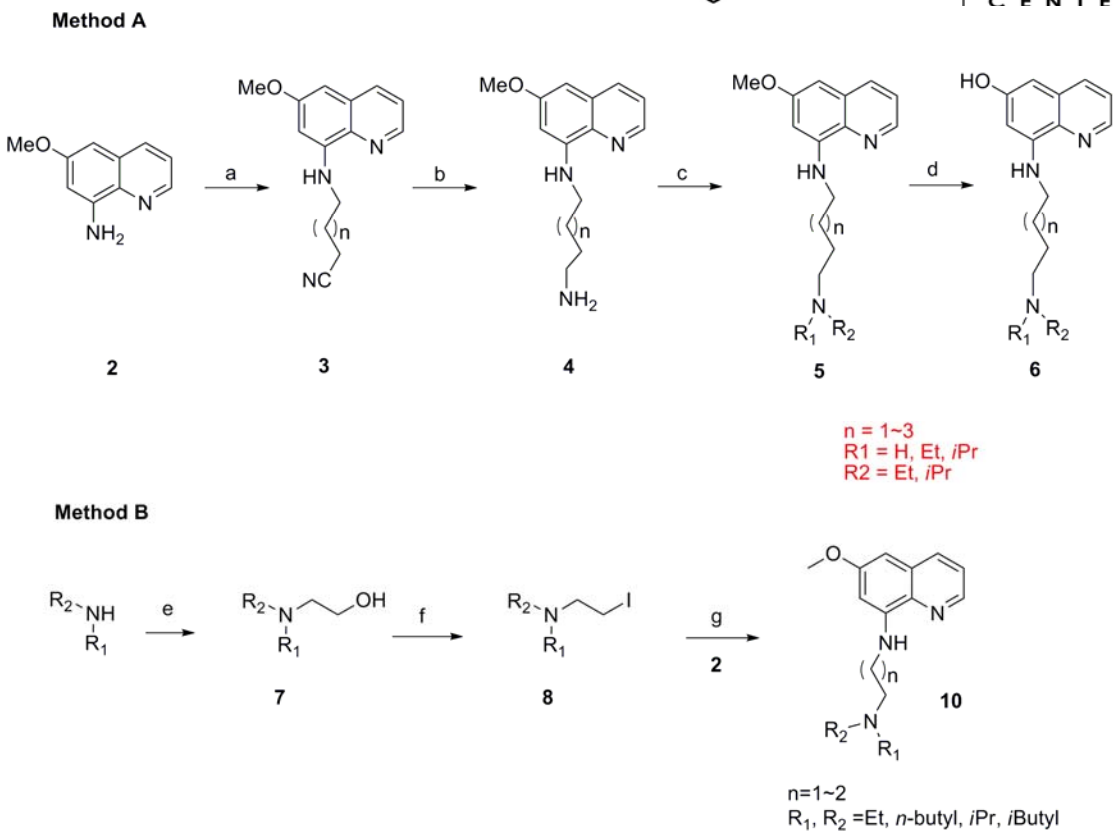
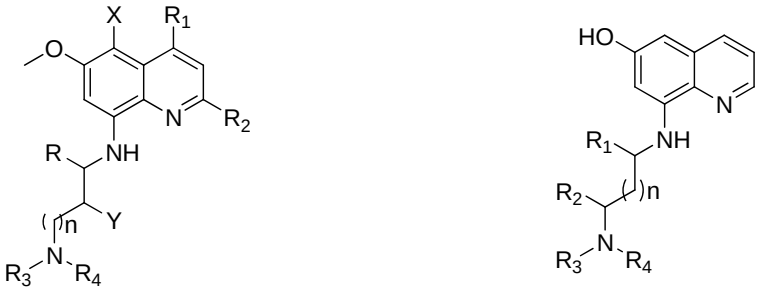


Fig. 2. Original Hsp90 hit compound **SID 850375** and active analogs.

- Detailed synthetic pathway for making probe: The activity of probe**1,** which was purchased from NCI, was confirmed by re-synthesis utilizing scheme 1. Some synthetic analogs around SID 850375 were also synthesized in an attempt to improve the activity (Scheme 1). The structures of SID 850375 and its derivatives are shown in **Fig. 2**.



Scheme 1. Representative synthetic scheme Reagents and conditions. **Method A**: a) $Br(CH_2)_2(CH_2)_nCN$, Et_3N, EtOH, refluxing, 3d; b) LAH, THF, 0°C-rt; c) CH_3CHO, $NaBH(OAc)_3$; d) HBr, reflux. **Method B**: e) Bromoethanol, Et_3N, THF, reflux; f) Imidazole 3eq, PPh_3 & I_2 (1.5 eq), Et_2O:AcCN; g) Et_3N, MeOH:DMF (2:1), 135 °C, 2 days.



10a: n = 0, R = R_1 = R_2 = H; R_3 = H, R_4 = *s*Bu; X = Y = H
10b: n = 0, R = R_1 = R_2 = H; R_3 = Et, R_4 = sPentyl; X = Y = H
10c: n = 1, R = R_1 = R_2 = H; R_3 = H, R_4 = *3*-pentyl; X = Y = H
10d: n = 2, R = R_1 = R_2 = H; R_3 = R_4 = H; X = Y = H
10e: n = 3, R = R_1 = R_2 = H; R_3 = H, R_4 = *t*-pentyl; X = Y = H
10f: n = 2, R = R_1 = Me, R_2 = H, R_3 = *n*-pr, R_4 = H; X = Y = H
10g: n = 2, R = Me, R_1 = R_2 = H, R_3 = *i*-pr, R_4 = H; X = Y = H
10h: n = 2, R = Me, X = OMe, R_1 = R_2 = Y = H, R_3 = *i*-pr, R_4 = H
10i: n = 2, R = Me, R_1 = R_2 = Y = H, X = *p*-Cl-PhO, R_3 = R_4 = H
10j: n=3, R = H, R_1 = R_2 = Y = H, X = *p*-Meo-PhO, R_3 = *i*-pr, R_4 = H
10k: n = 1, R = R_1 = R_2 = H, X = PhN, Y = H, R_3 = R_4 = Et
10l: n =1, R_1 = H, R_2 = OMe, X = Y = H, R_3 = R_4 = Et
10m: n =1, R_1= Cl, R_2 = H, X = Y = H, R_3 = R_4 = *i*-pr

6a: n = 1, R_1 = R_2 = H, R_3 = R_4 = H
6b: n = 1, R_1 = H, R_2 = Et, R_3 = R_4 = H
6c: n = 2, R_1 = R_2 = R_3 = R_4 = H
6d: n = 2, R_1 = H, R_2 = Me, R_3 = R_4 = H
1: n=3, R_1 = R_2 = H, R_3 = R_4 = Et
6e: n = 3, R_1 = H, R_2 = Me, R_3 = R_4 = H
6f: n = 3, R_1 = R = R_3 = R_4 = H
6g: n =1, R_1= Me, R_2 = H, R_3 = R_4 = Me
6h: n = 2, R_1 = Me, R_2 = H, R_3 = *iso*-propyl, R4 = H
6i: n = 2, R_1 = Me, R_2 = R_3 = R_4 = H
6j: n = 2, R_1 = Me, R_2 = H, R_3 = R_4 = Et

Fig. 3. Structures of quinoline analogs. Probe1 structure is denoted in blue.

- Center summary of probe properties (solubility, absorbance/fluorescence, reactivity, toxicity, etc.) and recommendations for the scientific use of probe as research tool: No significant properties have been measured experimentally for Probe**1**, but a selected variety of properties have been calculated using QikProp software (Jorgensen, 2004). None of the values of the properties calculated fall outside the 95% range of similar values for known drugs (Table 1), suggesting that probe**1** has favorable drug-like qualities. Probe**1** is completely stable under ambient conditions and as a solution in DMSO stored at -20°C. Since the molecule has no reactive functional groups, it would appear to be free of decomposition/reactivity/ toxicity problems *in vivo*.

Table1. Predicted ADME Properties for Probe**1**

ADME property	Property Description	Probe1 value	95% drug range
CNS	CNS activity	1	(-2 - 2)
dipole	Dipole moment	3.358	(1 - 12.5)
SASA	Total solvent accessible surfaced area; probe radius 1.4 Å	656.313	(300.0 - 1000.0)
volume	Total solvent accessible volume; probe radius 1.4 Å	1126.673	(500.0 - 2000.0)
QPlogPo/w	Predicted water/gas partition coefficient	3.331	(-2 - 6.5)
QPlogS	Predicted aqueous solubility, log S	-3.54	(-6.5 - 0.5)
QPlogHERG	Predicted IC_{50} vlue for blockage of HERG K+ channels	-6.486	(< -5)
QPPCaco	Apparent Caco-2 cell permeability in nm/sec	417.27	(< 25, poor; >500 great)
QPlogBB	Predicted brain/blood partition coefficient	-0.494	(-3.0 - 1.2)
QPPMDCK	Apparent MDCK cell permeability in nm/sec	212.788	(< 25, poor; >500 great)
QPlogKp	Predicted skin permeability	-3.416	(-8.0 - -1.0)
HumanAbs	Human Oral Absorption	3	(1=low, 2=med, 3=high)
QP%HumanAbs	% Human Oral Absorption	93.349	(>80% is high, <25 is poor)

Appendices

- Comparative data on probe, similar compound structures and prior probes: Probe**1** showed dose-dependent inhibition in the Hsp90 FP binding assay with an average *Ki* = 101.8 nM (95% confidence interval 44.7 nM – 231.9 nM) (**Fig.4**). The activity of commercial Probe**1** was confirmed by re-synthesis (**Fig.5**).

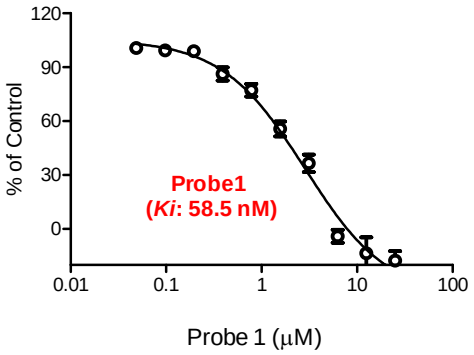


Fig. 4. Dose-response curve for the action of commercial probe **1** as determined by Hsp90 binding FP assay. Data shown are n = 1 (mean ± SEM of triplicate readings), representative of 5 independent

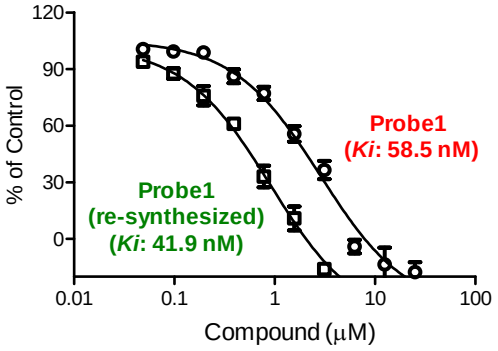


Fig. 5. The activity of Probe**1** in Hsp90 FP assay was confirmed by re-synthesis. Data shown are n = 1 (mean ± SEM of triplicate readings), representative of 5 independent experiments for Probe**1** and 3 independent experiments for re-synthesized probe**1**.

The dose-dependent effects of a total of 101 synthetic derivatives and 41 commercial compounds were tested in Hsp90 binding FP assay. The dose-curves of three representative analogs with different potencies relative to Probe**1** in the Hsp90 binding FP assay are shown in **Fig. 6**.

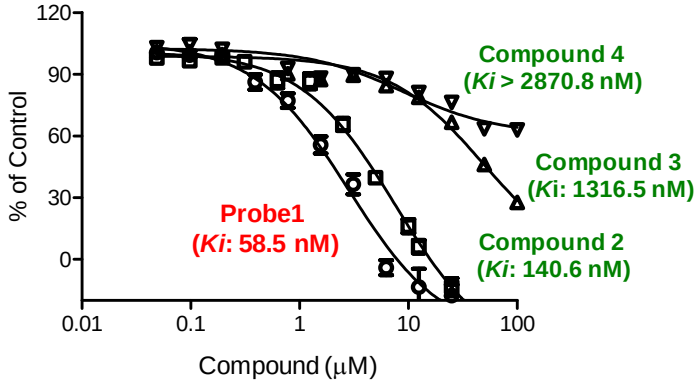


Fig. 6. The comparison of Probe**1** and three analogs in the Hsp90 FP assay. Data shown are n = 1 (mean ± SEM of triplicate readings), representative of 5 independent experiments for Probe**1**, 3 - 6 independent experiments for compounds 2, 3 and 4. Data are summarized in Table 2.

Analogs were tested further in the cell-based Her2 degradation assay in MCF-7 cells. Probe**1** showed dose-dependent degradation of Her2 protein with IC_{50} = 1.0 μM (95% confidence interval 0.4 μM – 2.3 μM) (**Fig. 7**). PU-H71 (PU) is a known Hsp90 inhibitor discovered by Dr. Gabriela Chiosis (Chiosis et al., 2008; He et al., 2006) and was used as a positive control in both the FP assay and the Her2 degradation assay. Three analogs of Probe**1** showed a similar pattern of Her2 protein degradation with different potencies (**Fig. 8**). The biological effects of probe**1** and three analogs in both the FP dose response and Her2 degradation assays are summarized in Table 2.

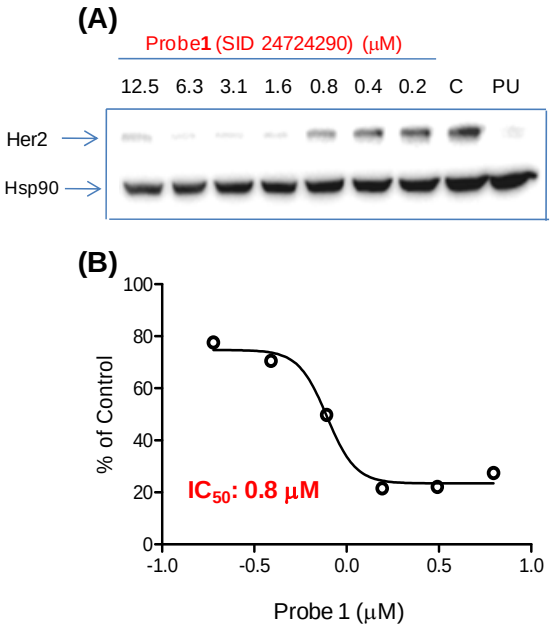


Fig. 7. Her2 degradation Western Blot assay for Probe**1**. (A) Probe**1** showed dose-dependent effect in degradation of Her2 protein as measured by Western blot assay. (B) Dose response curve of Probe**1** in Her2 degradation assay. Western Blot band density from (A) are quantified. Data are normalized to total Hsp90 protein level and then normalized to the vehicle (DMSO) control well. Data shown are n = 1, representative of 6 independent experiments. PU is the known Hsp90 inhibitor discovered by Dr. Gabriela Chiosis and used as a positive control (0.25 μM).

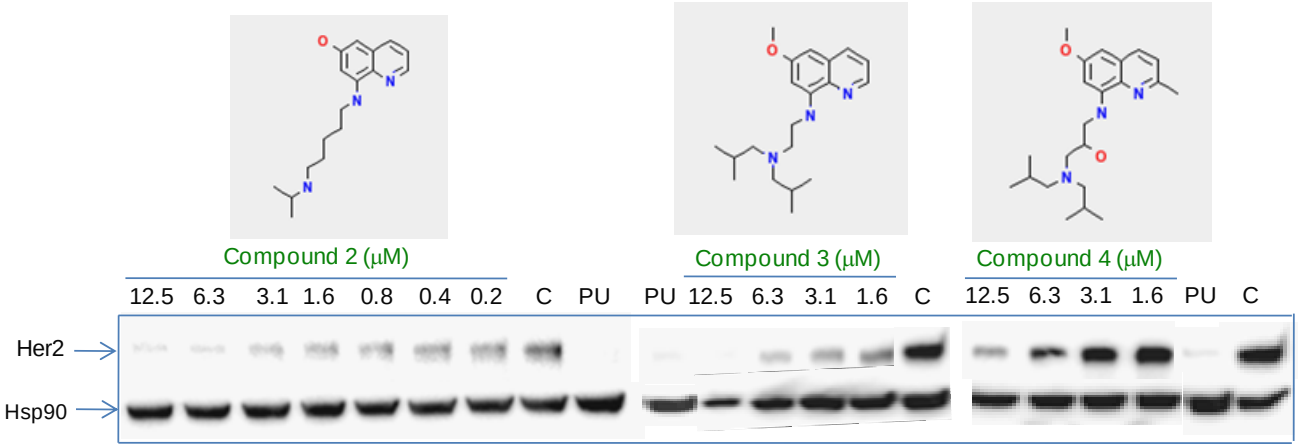


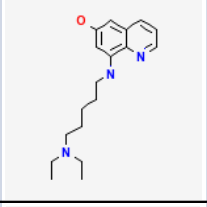
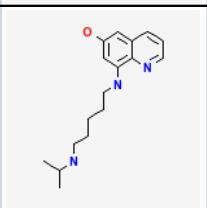
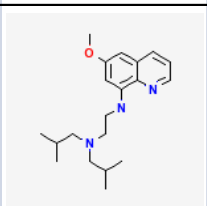
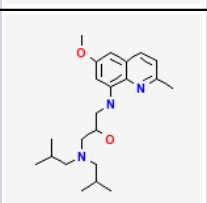
Fig. 8. Her2 degradation assay for Probe**1** analogs. Data shown are n = 1, representative of four independent experiments for compound2 and two independent experiments for compound 3 and compound 4. PU = PU-H71 used at 0.25 μM.

The proposed Probe1:Hsp90 interaction model is shown in **Fig. 9**. The GLIDE protocol (Schrodinger Inc.) was used to dock Probe**1** into the ATP binding pocket of Hsp90. Rendering for all pictures was generated using PyMol (DeLano Scientific). Docking results show that the compound makes contacts with key pharmacophoric centers observed in Hsp90 crystal structures, which is very similar with known Hsp90 inhibitors (**Fig. 10**).

The discovery of the first Hsp90 inhibitor, geldanamycin, led to increased interest in Hsp90 as a therapeutic target. However, the limited clinical efficacy of the geldanamycin derivative 17AAG, the first Hsp90 inhibitor to enter clinical trials, prompted further efforts to design improved, second-generation Hsp90 inhibitors (Solit and Chiosis, 2008). Such efforts have focused on both improving the formulation of existing Hsp90 inhibitors as well as the identification of novel scaffolds by direct chemical synthesis of purine-scaffold derivatives

(Chiosis et al., 2008) and small molecule library screening (Smith et al., 2006). Probe**1** is a novel scaffold that is easily synthesizable (Scheme 1) and is predicted to bind to Hsp90 in a manner similar to that of known Hsp90 inhibitors (**Figs. 9** and **10**). In addition, the potency of Probe**1** in both the FP dose response and Her2 degradations assays is close to that of several Hsp90 inhibitors currently in various stages of clinical development/trials, which exhibit 10 – 100 nM potency in cellular models. These results support the utility of Probe**1** in both *in vitro* and cell-based studies of Hsp90 function. Based on QikProp predictions, Probe**1** also has favorable "drug-like" properties (Table 2), including aqueous solubility and oral absorption. However, whether the selectivity and pharmacokinetic properties of Probe**1** are suitable for clinical development warrants further study.

Table 2. Summary of biological effects of Probe**1** and its analogs.

Compound No.	Structure	Compound (SID)	*Ki* (FP Assay) (nM)		IC50 (Western Blot) (uM)	
			mean *Ki* (nM)*	SD log *Ki*	mean IC_{50}	SD log IC_{50}
1		24724290	101.8	0.18	1.0	0.2
2		24724310	212.5	0.18	4.2	0.1
3		24724348	1177.7	0.21	3.4	0.1
4		24724372	NA	NA	4.9	0.2

NA: The IC_{50} > 50 μM in Hsp90 FP assay and *Ki* values is not available.

- <u>Comparative data showing probe specificity for target:</u> Probe**1** showed good correlation between Hsp90 binding and Her2 degradation, which is indicative of selective biological effect in cells via Hsp90. Moreover, the original hit compound (SID 850375) is only active in 7 primary HTS assay out of 175 assays tested in PubChem, suggesting that the behavior of the compound is selective across multiple assays.

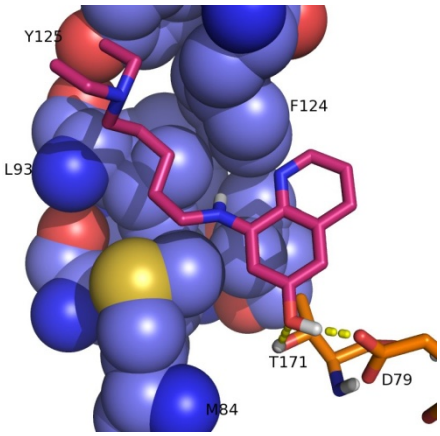


Fig. 9. Proposed Probe1:Hsp90 interaction model. Docking of the Probe1 (red) into the ATP N-terminal binding site of Hsp90. Docking results suggest that the compound makes contacts with key pharmacophoric centers observed in Hsp90 crystal structures. Hydrogen bonding contacts with probe**1** are represented with orange sticks (Hsp90 residues) and dotted yellow lines (hydrogen bonds).

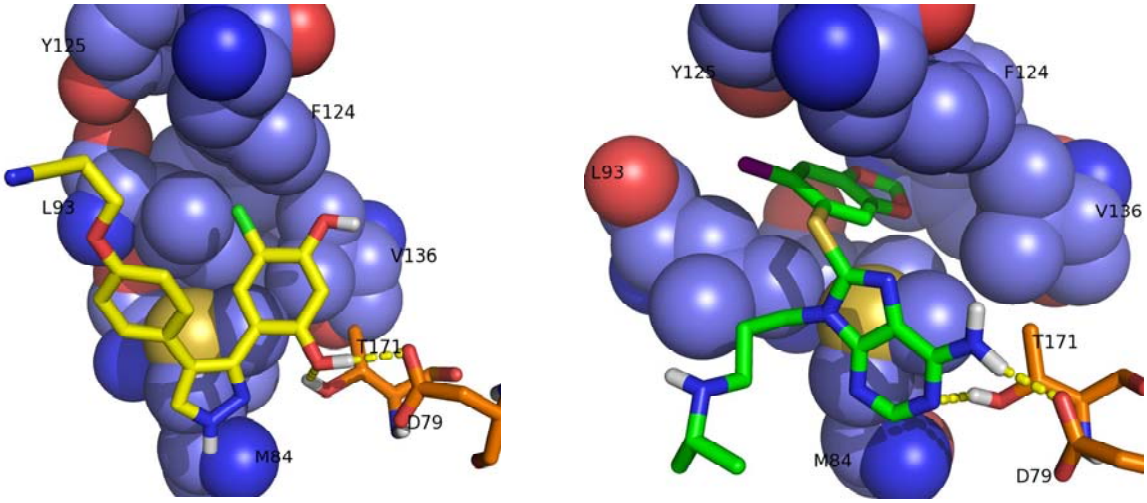


Fig. 10. **Crystallographic Poses of Known Hsp90 Inhibitors.** 3D representations of the hydrophobic and hydrogen bonding interactions with known pyrazole (yellow) and purine (green) Hsp90 inhibitors (PDB IDs: 2BRE, 2FWZ, respectively). Hydrophobic residues represented in blue spheres. Hydrogen bonding contacts with ligand are represented with orange sticks (Hsp90 residues) and dotted yellow lines (hydrogen bonds).

References

Chiosis G, Vilenchik M, Kim J, and Solit D. (2004) Hsp90: the vulnerable chaperone. *Drug Discov Today*. 9: 881-8.

Chiosis G, Kang Y, and Sun W. (2008) Discovery and development of purine-scaffold Hsp90 inhibitors. *Expert Opinion on Drug Discovery*. 3: 99-114.

DeLano, W.L The PyMol Graphics Systems (2002) on the World Wide Web http://www.pymol.org

Du Y, Moulick K, Rodina A, Aguirre J, Felts S, Dingledine R, Fu H, and Chiosis G. (2007) High-throughput screening fluorescence polarization assay for tumor-specific Hsp90. *J Biomol Screen*. 12: 915-24.

Glide, version 4.5, Schrodinger, LLC, New York, NY, 2007He H, Zatorska D, Kim J, Aguirre J, Llauger L, She Y, Wu N, Immormino RM, Gewirth DT, and Chiosis G. (2006) Identification of potent water soluble purine-scaffold inhibitors of the heat shock protein 90. *J Med Chem*. 49: 381-90.

Jorgensen WL. (2004) The many roles of computation in drug discovery. *Science*. 303: 1813-8.

Kamal A, Boehm MF, and Burrows FJ. (2004) Therapeutic and diagnostic implications of Hsp90 activation. *Trends Mol Med*. 10: 283-90.

Maloney A, and Workman P. (2002) HSP90 as a new therapeutic target for cancer therapy: the story unfolds. *Expert Opin Biol Ther*. 2: 3-24.

Neckers L, Schulte TW, and Mimnaugh E. (1999) Geldanamycin as a potential anti-cancer agent: its molecular target and biochemical activity. *Invest New Drugs*. 17: 361-73.

Nikolovska-Coleska Z, Wang R, Fang X, Pan H, Tomita Y, Li P, Roller PP, Krajewski K, Saito NG, Stuckey JA, and Wang S. (2004) Development and optimization of a binding assay for the XIAP BIR3 domain using fluorescence polarization. *Anal Biochem*. 332: 261-73.

Smith NF, Hayes A, James K, Nutley BP, McDonald E, Henley A, Dymock B, Drysdale MJ, Raynaud FI, and Workman P. (2006) Preclinical pharmacokinetics and metabolism of a novel diaryl pyrazole resorcinol series of heat shock protein 90 inhibitors. *Mol Cancer Ther*. 5: 1628-37.

Solit DB, and Chiosis G. (2008) Development and application of Hsp90 inhibitors. *Drug Discov Today*. 13: 38-43.

Xu W, Mimnaugh E, Rosser MF, Nicchitta C, Marcu M, Yarden Y, and Neckers L. (2001) Sensitivity of mature Erbb2 to geldanamycin is conferred by its kinase domain and is mediated by the chaperone protein Hsp90. *J Biol Chem*. 276: 3702-8.