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High-throughput screening for Hsp90 ATPase inhibitors

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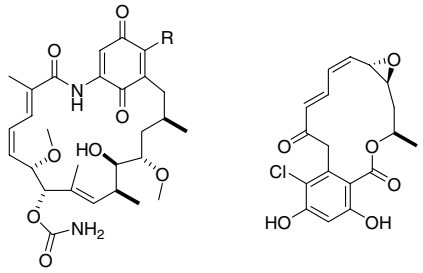
Abstract—Recently, we reported a useful assay for the determination of yeast Hsp90 ATPase activity. Using this assay, high-throughput screening of ~10,000 compounds was performed to determine the feasibility of this assay on large scale. Results from high-throughput screening indicated that the assay was reproducible (av *Z*-factor = 0.80) and identified 0.57% of the compounds as Hsp90 inhibitors that exhibited IC_{50}s less than 20 μM. The structures of several of these inhibitory scaffolds are reported along with their IC_{50} values.

The Hsp90 molecular chaperone is responsible for folding nascent polypeptides into biologically active structures and refolding denatured proteins back into their native conformation.[1–5] The Hsp90 protein folding process is driven by its inherent ATPase activity, which provides the requisite energy for the protein folding machinery to conformationally transform protein substrates.[6] Inhibitors of ATP binding/hydrolysis prevent Hsp90 from acquiring the energy necessary for this transformational process and produce unstable complexes that are ultimately degraded via the ubiquitin-proteasome pathway.[7] Therefore, small molecule inhibitors transform the normal protein folding machinery into a catalyst for protein degradation.

In contrast to traditional cancer chemotherapeutics, Hsp90 inhibitors have the potential to inhibit numerous signaling nodes that are mediated by the Hsp90 protein folding machinery. In fact, 48 oncogenic proteins have been shown to be dependent upon Hsp90 for conformational activation including telomerase, Her2 (erbB2), Raf-1, focal adhesion kinase, and the steroid hormone receptors.[8–13]

In 1994, geldanamycin (Fig. 1) was identified as the first natural product inhibitor of Hsp90.[14–17] The electrophilic nature of the quinone moiety and the redox-active behavior of this ring manifest toxicity unrelated to Hsp90 inhibition.[18] Radicicol, the most potent natural product inhibitor in vitro, is inactive in vivo.[19–21] In 2003, researchers reported that the geldanamycin derivative, 17-AAG, exhibits differential selectivity for cancer cells because Hsp90 in malignant cells forms a heteroprotein complex comprised of both client and partner proteins, which has a higher affinity for ATP than the homodimeric species found in nontransformed cells.[22] 17-AAG's poor solubility led to the development of 17-DMAG, which has been reported to be significantly more soluble than 17-AAG.[23–26] Both compounds have entered clinical trials for the treatment of cancer, however, there is a need to identify new inhibitory scaffolds that lack these detrimental



GDA, R = OMe
17-AAG, R = NHallyl
17-DMAG, R = $NH(CH_2)_2NMe_2$

RDC

Figure 1. Hsp90 inhibitors.

Keywords: Hsp90; High-throughput screening; Inhibitors.
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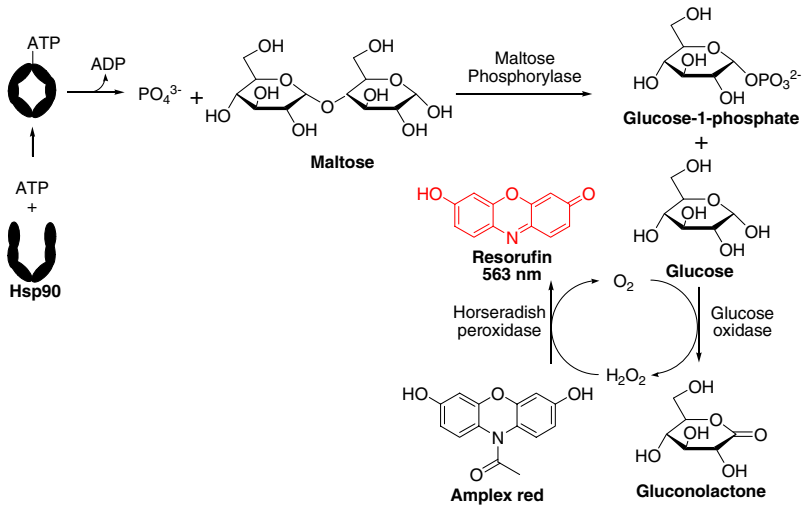

Figure 2. Mechanism of the coupled enzyme assay.

properties for the development of new cancer chemotherapeutics.

High-throughput screening is a useful method for the identification of novel inhibitory scaffolds.[27,28] Recently, we reported a coupled enzyme assay that was optimized for determination of yeast Hsp90's inherent ATPase activity, which still exists in the absence of protein substrates.[29] This assay utilizes maltose and amplex red as substrates for commercially available enzymes; maltose phosphorylase, glucose oxidase, and horseradish peroxidase. For every molecule of ATP that is hydrolyzed by yeast Hsp90, one molecule of amplex red is ultimately oxidized to resorufin, which has a unique absorption spectra at 563 nm (Fig. 2).

In an effort to determine whether this assay was suitable for high-throughput screening, we attempted to use it as previously described.[29] However, minor modifications were required before a reproducible *Z*-factor was obtained in a high-throughput setting utilizing 384-well plates.[30,31] These optimized conditions were used to screen approximately 10,000 compounds at The University of Kansas, High-Throughput Screening Laboratory.

Of the ~10,000 compounds screened in this assay, 81 molecules were shown to possess more than 75% inhibition at ~20 μM. The vast majority of compounds tested (>99%) produced little or no inhibition as demonstrated by the large number of squares (molecules) on the baseline of Figure 3. However, numerous compounds did show promising ATPase inhibition as illustrated by the squares found in the region corresponding to 100% inhibition.

Upon identification of molecules that diminished resorufin production, it was necessary to determine whether these molecules were selectively inhibiting Hsp90 or other components of the coupled assay system. Therefore, the compounds were evaluated in a subsequent assay in the presence of 100 μM inorganic phosphate, but in the absence of Hsp90.[32] Molecules that inhibited the formation of resorufin in the presence of inorganic phosphate were not selective for Hsp90. Of the 81 original hits, 54 compounds produced no effect in the control assay, indicating that these molecules selectively inhibit Hsp90.

Ten of the compounds identified from high-throughput screening were randomly chosen for further screening,

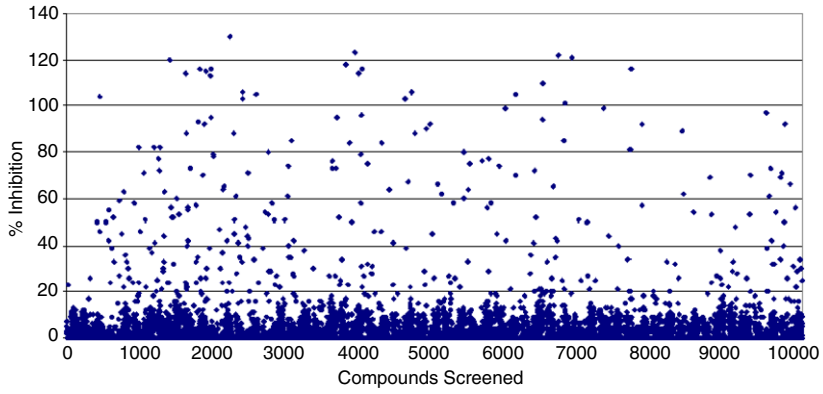

Figure 3. Scatter plot showing activities of the 10,000 screened compounds.

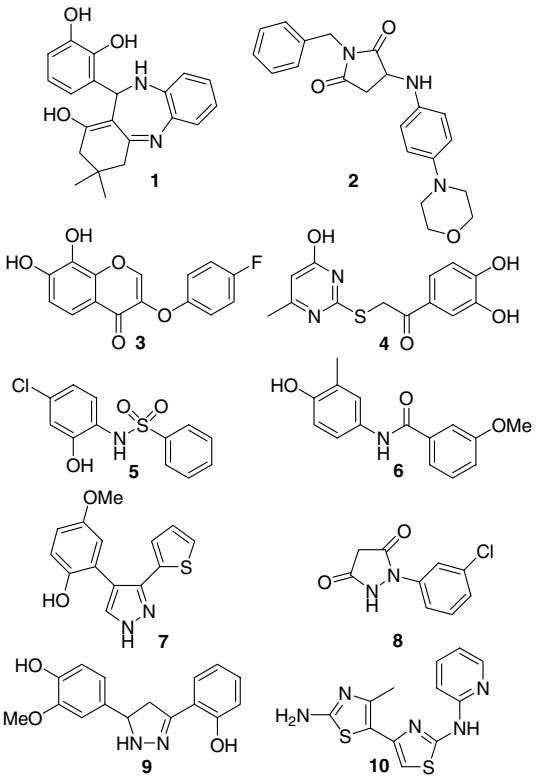

Figure 4. Ten compounds identified as Hsp90 inhibitors by HTS.

as seen in Figure 4. Not surprisingly, several of these structures resemble previously identified Hsp90 inhibitors including compounds **7**, **9**, and **10**.[33–35] IC_{50} values for each of these compounds were determined in the purified recombinant ATPase assay and are provided in Table 1. In this assay, all 10 of these compounds exhibited low micromolar activity ($IC_{50} < 7$ μM) and are comparable to geldanamycin, which produced an IC_{50} value of 2.5 μM.[29]

Seven of these scaffolds (**1–5**, **8**, and **9**) were selected for further evaluation. SKBr3 breast cancer cells were incubated with varying concentrations of each compound for 24 h, before Her2 levels were determined by ELISA.[36] Compounds **1**, **5**, and **9** were found to induce 50% degradation of the Hsp90-dependent client protein Her2, at concentrations of 61 ± 15, 31 ± 17, and 18 ± 5 μM, respectively.

The same compounds were subsequently screened for their ability to inhibit cell growth. Analogous to the Her2 results, only compounds **1**, **5**, and **9** exhibited anti-proliferative activity to produce IC_{50} values of 66 ± 24, 75 ± 13, and 48 ± 20 μM, respectively, which are similar to the IC_{50} values obtained from Her2 analysis.

In addition to Her2 ELISA studies, it was necessary to verify Her2 degradation along with another well-documented client, AKT, by Western blot analysis. Therefore, SKBr3 breast cancer cells were incubated with increasing concentrations of **1, 5**, and **9** for 24 h, before the cells were lysed and equal concentrations of protein probed for Her2, AKT, and actin. As can be seen in Figure 5, both proteins were degraded at concentrations that mirror the concentration needed to exhibit anti-proliferative activity. Since actin is not an Hsp90 substrate, it was used as a negative control to verify that all protein levels were not affected by these inhibitors.

From the Her2 ELISA studies, three compounds (**1**, **5**, and **9**) emerged as the only compounds that induced significant Her2 degradation. IC_{50} values obtained from cell growth assays were similar to those obtained from Western blot analysis of Hsp90 clients Her2 and AKT, thereby linking Hsp90 inhibition directly to anti-proliferative activity. Previous studies have shown that the concentration of Hsp90 inhibitors needed to induce Her2 degradation mirrors that needed to impair cell proliferation.[37] Compounds that did not induce Her2 degradation (via Her2 ELISA) or affect proliferation are potentially unable to diffuse through the lipid bilayer or may bind other proteins/enzymes, thus lowering the amount of available compound to bind Hsp90.

Table 1. IC_{50}s for compounds **1–10**

Compound	IC_{50}[a] (μM)		Antiproliferation IC_{50} (μM)
	Hsp90 ATPase inhibition	Her2 ELISA	
1	2.7	61 ± 15	66 ± 24
2	1.9	na	na
3	3.2	na	na
4	2.3	na	na
5	2.0	31 ± 17	75 ± 13
6	2.5	na	na
7	3.0	na	na
8	4.1	na	na
9	5.4	18 ± 5	48 ± 20
10	7.0	na	na

[a] Values are means of two experiments ± SD performed in triplicate, (na, not active).

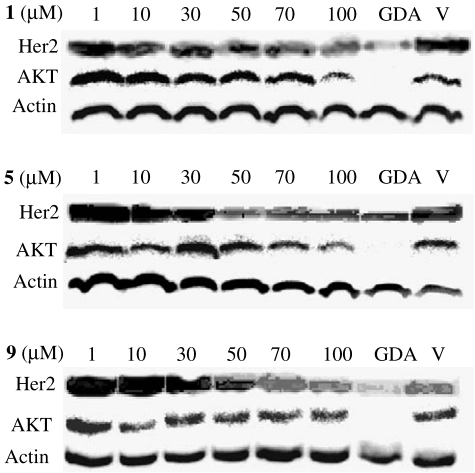

Figure 5. Western blot analyses for **1**, **5**, and **9**. GDA = 0.5 μM, V = vehicle control (1% DMSO).

Although the original ATPase assay required modification before implementation in a high-throughput setting, the optimized assay proved to be reliable for identification of new Hsp90 inhibitors. The *Z*-factor obtained from these assays averaged 0.80, which indicates the assay is highly reproducible. Of the ~10,000 compounds screened, 81 compounds (0.81%) were identified as inhibitors that produced >75% inhibition at 20 μM. Approximately 1/3 of these compounds were not selective for Hsp90, leaving 54 compounds (0.54%) as novel small molecules selective for Hsp90 inhibition. Some of the molecules identified by high-throughput screening with this assay had similar structures to those previously reported, however, new scaffolds such as **1**–**6** and **8** have been identified as new motifs that inhibit Hsp90 ATPase activity and may serve as lead compounds for further drug discovery efforts. Furthermore, results from this screening experiment validate the potential for this assay in a much larger high-throughput screening effort.

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