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An AlphaScreen™-Based High-Throughput Screen to Identify Inhibitors of Hsp90-Cochaperone Interaction

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Hsp90 has emerged as an important anticancer drug target because of its essential role in promoting the folding and maturation of many oncogenic proteins. The authors describe the development of the first high-throughput screen, based on AlphaScreen™ technology, to identify a novel type of Hsp90 inhibitors that interrupt its interaction with the cochaperone HOP. The assay used the 20-mer C-terminal peptide of Hsp90 and the TPR2A domain of HOP. Assay specificity was demonstrated by measuring different interactions using synthetic peptides, with measured IC₅₀s in good agreement with reported values. The assay was stable over 12 h and tolerated DMSO up to 5%. The authors first validated the assay by screening against 20,000 compounds in a 384-well format. After further optimization into a 1536-well format, it was screened against an NIH Chemical Genomics Center library of 76,134 compounds, with a signal-to-background ratio of 78 and Z' factor of 0.77. The present assay can be used for discovery of novel small-molecule Hsp90 inhibitors that can be used as chemical probes to investigate the role of cochaperones in Hsp90 function. Such molecules have the potential to be developed into novel anticancer drugs, for use alone or in combination with other Hsp90 inhibitors. (*Journal of Biomolecular Screening* 2009;273-281)

Key words: heat shock protein 90 (Hsp90), Hsp organizing protein (HOP), tetratricopeptide repeat (TPR), AlphaScreen™, high-throughput screening (HTS)

INTRODUCTION

HHEAT SHOCK PROTEIN 90 (Hsp90) HAS EMERGED as an important anticancer drug target due to its essential activity for the folding and maturation of multiple oncogenic signaling proteins that regulate cancer cell growth and survival.¹ Specific inhibition of Hsp90 function leads to the destabilization and eventually the proteasomal degradation of multiple Hsp90 client proteins on which cancer cell growth is highly dependent.² To date, studies on developing small-molecule inhibitors of Hsp90 have exclusively focused on inhibiting its ATPase activity, using both structure-based design³ and high-throughput screening (HTS).⁴ Geldenamyacin (GM) and its analog 17-allylamino-geldanamycin (17-AAG), which act by competitively binding to the adenosine triphosphate/adenosine diphosphate (ATP/ADP)

pocket at the N-terminal domain of Hsp90, are particularly well-characterized examples of such inhibitors. 17-AAG has entered clinical trials in treating cancer patients.⁵ However, 17-AAG suffers from many limitations in administration (e.g., limited oral bioavailability, poor solubility, and liver toxicity).^{6,7} There is clearly a need to develop new Hsp90 inhibitors that have better pharmacologic and toxicity profiles than 17-AAG and function by a different mechanism.

Hsp90 does not function alone but interacts with a cohort of cochaperones to form multichaperone complexes.^{8,9} To our knowledge, however, there have been no prior reports of small molecules that inhibit Hsp90 by preventing its interaction with cochaperones. Here we report the development, optimization, and implementation of the first in vitro HTS to identify small-molecule compounds that disrupt the interaction between Hsp90 and its cochaperone Hsp90/Hsp70-organizing protein (HOP). HOP mediates the assembly of Hsp70-HOP-Hsp90 multichaperone complex through the interaction between its individual tetratricopeptide repeat (TPR) domains and the C-terminal peptides of Hsp70 and Hsp90.¹⁰ The core contact for the Hsp90 and HOP interaction has been identified to be between the C-terminal pentapeptide of Hsp90 (MEEVD) and the TPR2A domain of HOP, with a binding stoichiometry of 1:1 and a dissociation constant (K_d) in the micromolar range.^{11,12} Exogenous TPR domains act as dominant negative mutants when introduced into cells, leading to decreased activity and levels of Hsp90-dependent client proteins, presumably by

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competing with endogenous HOP and/or other TPR-containing proteins for their interactions with Hsp90.^{13,14} These observations provide a strong rationale for our approach to seeking small molecules that inhibit Hsp90 function by preventing the Hsp90-HOP interaction.

We have developed an HTS assay based on the Amplified Luminescence Proximity Homogeneous Assay (AlphaScreen™) technology to identify small molecules that inhibit the Hsp90-TPR2A interaction. We first demonstrate the specificity and utility of this assay by showing that it can distinguish between the interactions of the TPR2A domain with its cognate and noncognate ligands, C-terminal peptides of Hsp90 and Hsp70. When tested against 20,000 chemical compounds in a 384-well format, the assay has an average signal-to-background (S/B) ratio of 60 and Z' factor of 0.76, indicating the suitability of this assay for HTS. We further optimized the assay into a 1536-well assay format and screened against a collection of 76,314 compounds at the NIH Chemical Genomics Center (NCGC). Here we present the detailed description of assay development, HTS, and the "hit" compounds that we identified, which were further characterized in other follow-up assays. Such novel small-molecule Hsp90 inhibitors will be useful not only as molecular probes to aid in understanding the functional significance of interactions between Hsp90 and its cochaperones but also have the potential to be developed into novel anticancer drugs, to be used alone or in combination with other chemotherapy agents.

MATERIALS AND METHODS

Reagents for AlphaScreen™ assay

N-terminal His₆-tagged TPR2A was produced using a bacterial expression system and purified using Ni-NTA superflow resin (Qiagen, Valencia, CA). C-terminal peptides of Hsp70 (FGAQGPKGGSGS-GPTIEEVD) and Hsp90 (TEEMPPLEGDD-DTSR-MEEVD) with or without an N-terminal biotin group were synthesized using automated solid-phase synthesis by the Yale Keck facility. The reaction buffer was 25 mM HEPES (pH 7.4), 100 mM NaCl, and 0.1% bovine serum albumin (BSA) was added to minimize nonspecific interaction between beads. HEPES, NaCl, and BSA were purchased from Sigma (St. Louis, MO). Opti-384 plates and an AlphaScreen™ Histag fusion detection 10k assay point kit, which includes streptavidin-coated Donor beads and nickel-chelated Acceptor beads; the TrueHits 1k assay point kit, which includes streptavidin-coated Donor beads and biotinylated Acceptor beads; and the Biotin-His₆ linker peptide for the counterscreen were purchased from PerkinElmer (Montreal, Quebec, Canada). We chose the nickel-chelated acceptor beads over the anti-histag antibody-coated ones based on the consideration that the antibody, upon binding to the His₆-tagged TPR2A, might cause steric hindrance and interfere with the interaction between the TPR2A protein and Hsp90 peptide. In addition, 1536-well

polypropylene clear plates (Kalypsys, San Diego, CA) were used as compound plates, and 1536-well polystyrene white plates (solid bottom) from Corning, Inc. (Corning, NY) were used as assay plates.

Molecular libraries

The compounds tested in this study included 20,000 compounds with diverse chemical structures from Maybridge, Inc. (Cornwall, UK) and a NCGC library of 76,174 compounds. All compounds were stored at a stock concentration of 10 mM in 100% DMSO in 384-well plates at -80 °C.

Assay development and characterization in a 384-well format

For the initial assay characterization, the AlphaScreen™ competition assay was performed in white 384-well Opti plates under the following conditions: 10 nM biotin-Hsp90 peptide, 100 nM His₆-tagged TPR2A protein, and competitors such as free Hsp90 or Hsp70 peptides at different concentrations were incubated together for 1 h. Donor and Acceptor beads were then added to a final concentration of 10 µg/ml in 25 µl buffer containing 25 mM HEPES (pH 7.4), 100 mM NaCl, and 0.1% BSA. In the assay, the biotin-Hsp90 peptide was attached to streptavidin-coated Donor beads, and the His₆-tagged TPR2A was attached to nickel-chelated Acceptor beads. Nonbiotinylated free Hsp90 or Hsp70 C-terminal peptides served as control inhibitors by competing with biotinylated peptide for the binding to TPR2A without bringing the beads together.

The effect of DMSO, a common solvent for small-molecule compounds used for HTS, on assay signal was determined by incubating the assay reaction (25 µL, 10 nM biotin-Hsp90 and 100 nM His₆-tagged TPR2A, and 10 µg/mL beads) in different wells at various DMSO concentrations and read at the same time. Competition experiments by free Hsp90 peptide were also performed at 0%, 2.5%, and 5% DMSO (v/v) to evaluate the solvent effect on the IC₅₀ values.

Free Hsp90 peptide was used as a control inhibitor to test whether its competition for the interaction between biotin-Hsp90 and His₆-tagged TPR2A was reversible and how long it took for the competition to reach equilibrium. A "disaggregation" experiment was performed, in which the biotin-Hsp90, His₆-tagged TPR2A protein, and the Donor and Acceptor beads were preincubated in a stock solution for 1 h; 20 µL of the reaction was then aliquoted into individual wells in a 384-well plate, and the reaction was initiated by adding 10 µM free Hsp90 peptide in each well. This concentration was chosen because that was the reported K_d for the Hsp90-TPR2A interaction,^{11,12} and it was about the final compound concentration to be used in the HTS screen. Individual wells were read at different time points to avoid photo-bleaching.

Considering the time (~8.5 min) the plate reader takes to read one 384-well plate in the AlphaScreen™ mode, we tested

the stability of the assay signals using 2 control populations, positive controls and negative controls. We measured the concentration-response curves for free Hsp90 competition at time periods between 0.5 and 24 h after the addition of Donor and Acceptor beads. A replicate plate was used for each time point to avoid photo-bleaching. The signals were stable up to at least 12 h, whereas an incubation time of 24 h resulted in ~30% signal decrease in the maximum, leading to lower S/B ratios. There were no significant differences between the IC₅₀ values at different time periods. These results indicate that we can read at least 85 plates (384 well) over a 12-h period in 1 experiment.

Assay development into a 1536-well format

We further optimized the assay conditions to miniaturize the assay into a 1536-well plate format by including in the assay buffer a reducing reagent, dithiothreitol (DTT), which was originally left out of the assay with the concern that it might interfere with the nickel that is chelated to the Acceptor beads. Adding DTT significantly increased the assay signal, suggesting that potential disulfide bond formation by the 2 Cys residues in TPR2A may change the protein conformation and subsequently reduces its binding affinity to the Hsp90 C-terminal peptide. We also examined the effects of salts on the assay. Although the addition of K⁺ or Na⁺ in the assay buffer both produced robust signals, the assay was more sensitive in the K⁺-containing buffer. In addition, it is known that intracellular K⁺ concentration is approximately 100 mM, whereas Na⁺ is 10 mM. Thus, we selected 100 mM K⁺ to use in the assay buffer. In the final optimized 1536-well plate assay, the total assay volume was 5 µL. Briefly, 3 µL/well of His₆-tagged TPR2A (5 nM final) and 1 µL biotin-Hsp90 (10 nM final) were dispensed to a white 1536-well plate followed by an addition of 20 nL compound diluted in DMSO and a 2-h incubation. Subsequently, 1 µL/well of bead mixture containing both Acceptor and Donor beads was added. After a 1-h incubation at room temperature, the assay plate was measured in a PerkinElmer Envision plate reader. In each 1536-well plate, His₆-tagged TPR2A and biotin-Hsp90 were added to the first 2 columns as positive control. Columns 3 and 4 contained the His₆-tagged TPR2A, biotin-Hsp90, and free Hsp90 peptide as negative control for the basal signal. The remaining wells, the sample area, could contain up to 1408 compounds.

TrueHits kit screen and counterscreen using a Biotin-His₆ peptide

In the TrueHits kit screen, the strong binding between streptavidin and biotin brings the Donor and Acceptor beads directly together to generate signals upon excitation at 680 nm under all conditions. In the counterscreen, a Biotin-His₆ peptide serves as a covalent linker to bring together the streptavidin-coated Donor beads and nickel-chelated Acceptor beads. Any

compound that causes decreased signals in the TrueHits kit screen or the counterscreen must be general interfering compounds that are not relevant to the specific target of interest, whereas those that exhibit no effect on the signal are potential true hits. Donor beads and Acceptor beads (final concentration of 10 µg/mL) and each primary “hit” compound were incubated together with (for counterscreen) or without (for TrueHits kit screen) the linker peptide at room temperature for 1 h before the measurement. The screens were performed in the same assay buffer as that used in the HTS.

Data analyses

Data analysis for the 20,000-compound screen and following IC₅₀ measurements at the Yale Chemical Genomics Screening Facility was performed using Prism software (Graphpad Software, Inc., San Diego, CA). The concentration-response (CR) screening results from the NCGC screen of 76,174 compounds were analyzed using the NCGC CurveFit software (www.ncgc.nih.gov). A 4-parameter Hill equation was fitted to the CR data by minimizing the residual error between the modeled and observed responses. Outliers were masked if the difference with the modeled Hill equation exceeded the noise in the assay, which was calculated from the standard deviation of the activity at the lowest tested compound concentration. In addition, data from higher concentrations were preferentially masked if doing so allowed the fit of the lower concentration data to achieve significance, as judged by efficacy and *r*² requirements. Concentration-response curves were plotted using Prism software. The Z' factor, an index for assay quality control, was determined by

$$Z' = 1 - (3 * SD_{high} + 3 * SD_{low}) / (\text{Mean}_{high} - \text{Mean}_{low}).$$

All values are expressed as mean ± SD.

RESULTS AND DISCUSSION

Characterization of AlphaScreen™ competition assay to measure different protein-ligand interactions

AlphaScreen™ is a beads-based assay that relies on the proximity of “Donor” and “Acceptor” beads conjugated to different biomolecules of interest.^{15,16} The streptavidin-coated Donor beads and nickel-chelated Acceptor beads are brought together through the biotin-Hsp90/His₆-tagged TPR2A interaction (Fig. 1). Upon laser excitation at 680 nm, the photosensitizers inside the Donor beads convert ambient oxygen to a singlet oxygen state. Only when the Donor and Acceptor beads are brought within 200 nm can the singlet oxygen molecules diffuse to the Acceptor beads, resulting in extensive emission at 520 to 620 nm. To demonstrate appropriate binding and the sensitivity of the assay, we titrated His₆-tagged TPR2A protein and biotin-Hsp90 peptide to determine the optimal reagent concentrations (Fig. 2). At each of the 3 TPR2A concentrations tested, a plateau

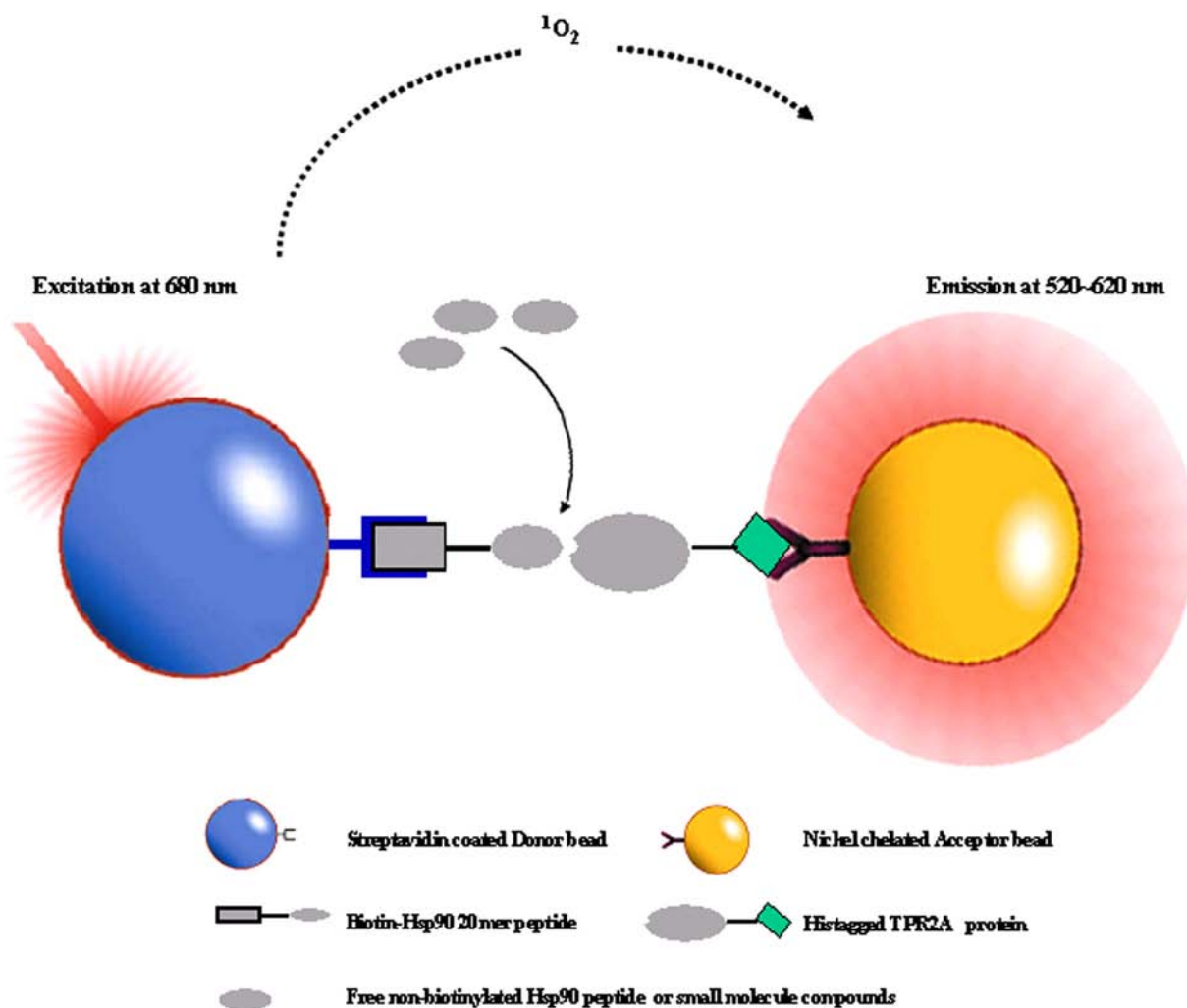


FIG. 1. Schematic diagram of AlphaScreen™-monitored Hsp90 competition assay. The biotin-Hsp90 peptide and His₆-tagged TPR2A interaction brings the beads together by conjugating to streptavidin-coated Donor beads and nickel-chelated Acceptor beads, respectively. Upon excitation at 680 nm, Donor beads produce singlet oxygen, which diffuses to Acceptor beads within 200 nm, initiating a chemiluminescent reaction. Any nonbiotinylated molecules (peptides or compounds) that can bind to TPR2A will inhibit the signals by competing with the biotinylated Hsp90 peptide.

of maximum signal was reached at ~50 nM biotin-Hsp90, indicating the Donor beads became saturated by biotin. A TPR2A concentration of 3.25 nM gave a maximum signal of only 2000 and an S/B ratio of 4 and thus was too low for the following characterization, whereas the S/B ratios for the binding using 325 nM and 32.5 nM His₆-tagged TPR2A were 144 and 133, respectively. With the intention to keep the biotin-Hsp90 peptide concentration as low as possible for the competition assay while still maintaining a high assay signal and sensitivity, we used 10 nM biotin-Hsp90 peptide and 100 nM His₆-tagged TPR2A, with an estimated S/B ratio around 60, for the following assay characterization.

We then assessed the kinetics of the binding reaction that gave rise to the AlphaScreen™ signal. Time course experiments

demonstrated that maximal signal was reached 30 min after incubating the assay components (**Fig. 3A**). Fitting the data to a single-phase exponential decay gave a half-life for the disaggregation reaction of 35 min (**Fig. 3B**). This represented the upper limit for the equilibration time necessary for the competition assays because in this experiment, the free Hsp90 peptide was added after the bead complexes were preincubated, whereas in the actual screen, the compound competitors were added at the same time as the biotin-Hsp90 peptide. Based on these results, biotin-Hsp90, compounds, and TPR2A were incubated for at least 1 h to ensure the competition reached equilibrium before addition of the beads.

The C-terminal peptides of Hsp70 and Hsp90 serve as test peptides to assess the specificity of the AlphaScreen™ competition

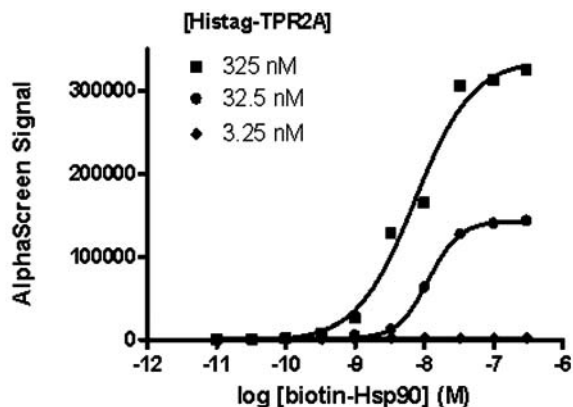


FIG. 2. Dose-dependent binding of biotinylated Hsp90 C-terminal peptide to TPR2A. Increasing concentrations of biotin-Hsp90 peptide were incubated with 325 nM (■), 32.5 nM (●), or 3.25 nM (◆) His₆-tagged TPR2A and Acceptor beads (20 μg/mL) for 1 h. Donor beads were then added into each well to a final concentration of 20 μg/mL in a 25-μL assay volume. After a 1-h incubation, the plate was read on an Envision plate reader.

assay. Competitive displacement measurements were performed using the free 20-mer Hsp90 and Hsp70 C-terminal peptides, with IC₅₀s of 4.9 μM and 24 μM, respectively (Fig. 4), which are consistent with the reported values.¹¹ Multiple independent measurements yielded an average IC₅₀ of 4.4 μM with a standard

deviation of 2.2 μM for the Hsp90-TPR2A interaction. A C-terminally amidated Hsp90 peptide, deficient in binding due to its inability to form a “2-carboxylate clamp” with TPR2A,¹¹ did not compete with biotin-Hsp90 for TPR2A binding in this assay (data not shown). These results validate the use of this assay to identify small-molecule compounds that disrupt the Hsp90-HOP interaction.

Optimization of the assay

We tested the effect of DMSO on the AlphaScreen™ signal, S/B ratio, and IC₅₀ for competition curves at concentrations up to 5%. DMSO decreased the signals in a concentration-dependent manner, without affecting the IC₅₀ values of the competition curves. The S/B ratios were 51, 42, and 35 for 0%, 2.5%, and 5% DMSO concentration, respectively (data not shown). The actual HTS was performed in the presence of 0.1% DMSO.

We also explored the possibility of lowering the bead concentration to lower the assay cost. The effect of different bead concentrations (20, 10, 7.5, 5 μg/mL) on IC₅₀, assay signal, and S/B ratio was tested using the same peptide and protein concentration. The signal decreased with decreased bead concentration in a dose-dependent but not a linear way. The IC₅₀ values of the competition curves at different bead concentrations were the same within measurement error (data not shown). To ensure a minimum signal >20,000, we used a bead concentration of 10 μg/mL, with a maximum signal of over 100,000 and an S/B ratio of 130.

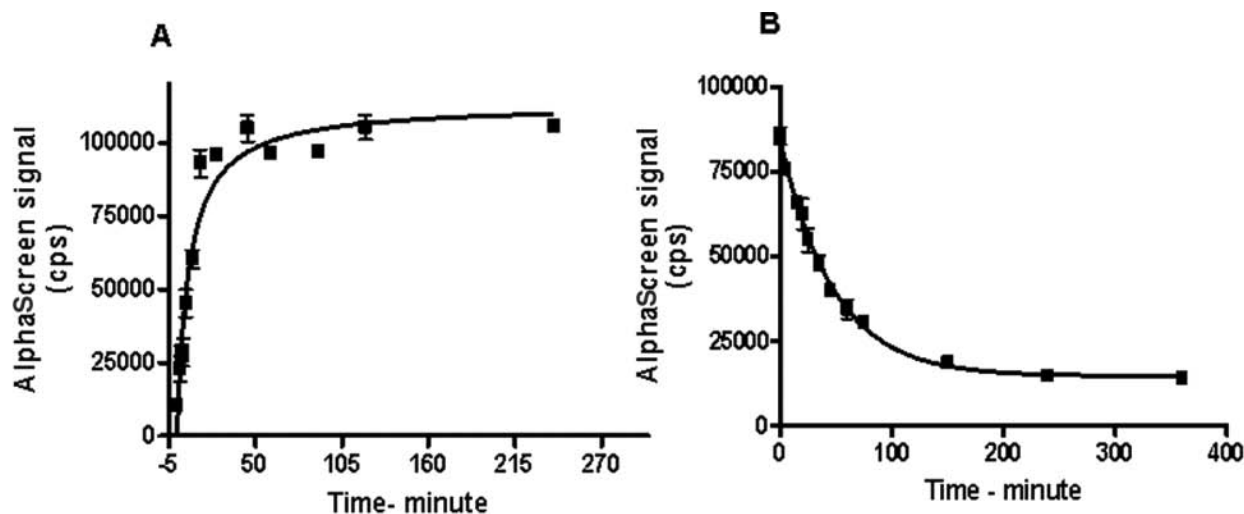


FIG. 3. (A) Time course of the biotin-Hsp90 peptide binding to His₆-tagged TPR2A protein. His₆-tagged TPR2A (100 nM) and 10 μg/mL Donor and Acceptor beads were incubated for 1 h. The reaction was initiated by adding 10 nM biotin-Hsp90 peptide, and measurements were taken at different time points. A different well was used for each time point to avoid photo-bleaching. Data are expressed as mean ± SD from 3 experiments. (B) Time for the competition assay to reach equilibrium. His₆-tagged TPR2A (100 nM), biotin-Hsp90 peptide (10 nM), and beads (10 μg/mL) were incubated for 1 h before the addition of 10 μM free Hsp90 peptide. A different well was used for each time point to avoid photo-bleaching. Data are expressed as mean ± SD from 3 experiments performed in triplicate.

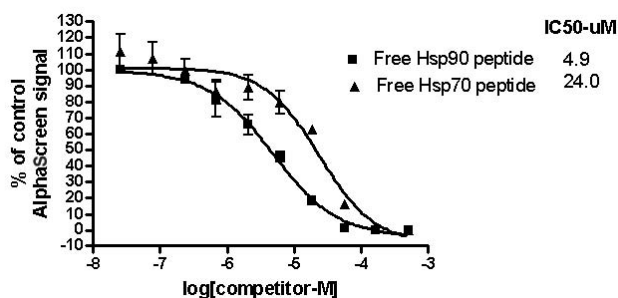


FIG. 4. Competition of free nonbiotinylated Hsp90 and Hsp70 peptides with biotinylated Hsp90 peptide for binding to the TPR2A protein. Biotinylated Hsp90 peptide (10 nM) and TPR2A protein (100 nM) and increasing concentrations of free Hsp90 (■) or Hsp70 (▲) peptides were incubated for 1 h first, and then the reaction was initiated by addition of 10 $\mu\text{g}/\text{mL}$ Donor and Acceptor beads. The plate was read after a 1-h incubation. The y-axis represents the percentage inhibition of the maximal AlphaScreen™ signal in the absence of any inhibitor. Data are expressed as mean \pm SD from 3 experiments.

Robustness of the AlphaScreen™ assay

To evaluate the suitability of the Hsp90 competition assay for HTS, we studied the intraplate variability on 2 control populations: the positive controls (10 nM biotin-Hsp90, 100 nM His₆-tagged TPR2A, 10 $\mu\text{g}/\text{mL}$ Donor and Acceptor beads in the absence of any inhibitors) and the negative controls

(10 nM biotin-Hsp90, 100 nM His₆-tagged TPR2A, 10 $\mu\text{g}/\text{mL}$ Donor and Acceptor beads with 30 μM free Hsp90 peptide). We used 48 wells in a 384-well plate to measure the positive controls and 48 different wells to measure the negative controls (Fig. 5). The average signal for positive controls was 121,833 with a standard deviation of 8118 (coefficient of variation [CV] = 6.7%); the average signal for negative controls was 2022 with a standard deviation of 442 (CV = 21.9%). The S/B ratio obtained was 60. Interday variability was around 11%. The Z' factor was 0.76 for the 2-day measurements, indicating the good quality of the assay. For day-to-day variance measurements, a replicate plate was read on a different day to avoid photo-bleaching.

HTS of compound libraries

In total, 20,000 compounds were screened at a single concentration of $\sim 10 \mu\text{M}$ at the Yale Chemical Genomics Screening Facility using the optimized 384-well assay format. Using a selection criterion of >50% inhibition at $\sim 10 \mu\text{M}$ compound, 60 primary “hits” were identified from this library.

In total, 76,174 compounds were screened at the NCGC using the optimized assay in quantitative HTS (qHTS) in the 1536-well format. Each compound was titrated in 7 to 15 concentrations, generating CR curves. This titration format, rather than single-concentration screening with follow-up CR curve measurements, allowed us to more efficiently select the hits and quickly narrow them down to a small group of active compounds for follow-up study.¹⁷ We included 0.1% BSA and 0.01% Tween-20 in the

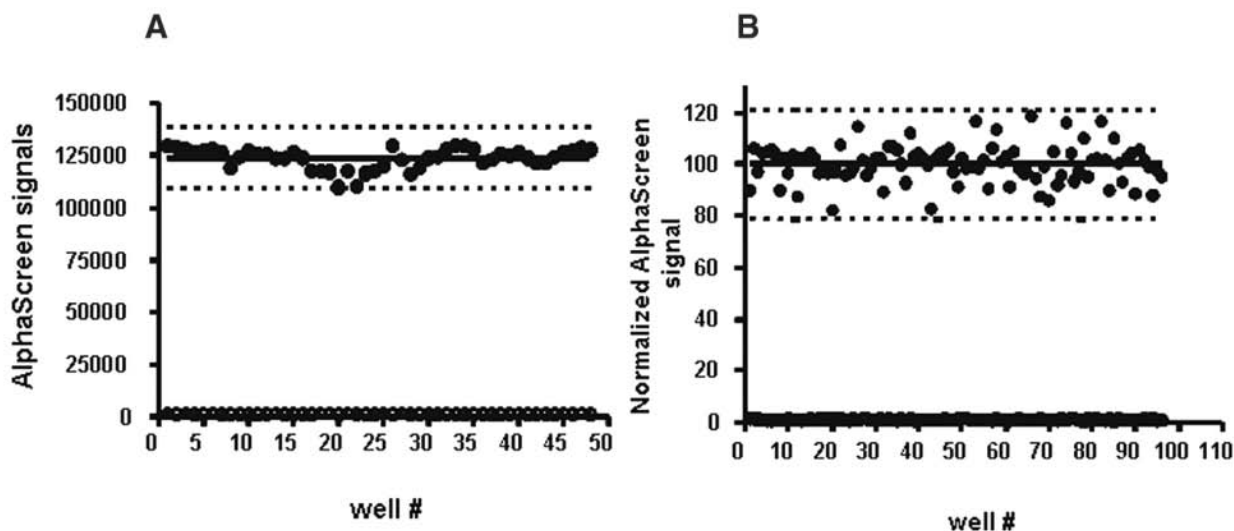


FIG. 5. High-throughput assay format evaluation using 2 control populations. (A) Intraplate variance using 48 positive controls (●, 10 nM biotin-Hsp90, 100 nM histag-TPR2A, and 10 $\mu\text{g}/\text{mL}$ beads) and negative controls (○, 10 nM biotin-Hsp90, 100 nM histag-TPR2A, and 10 $\mu\text{g}/\text{mL}$ beads in the presence of 30 μM free nonbiotinylated Hsp90 peptide) in one 384-well plate. The lines represent the mean (solid) and 3 standard deviations (dashed) from the mean of the positive and negative controls. (B) Interday variation. Two plates with samples from the same preparation were incubated and read at 2 different days. Data are expressed as percentage of maximum signals by normalizing against the average of positive controls in each plate. Data were plotted by row first, then by column.

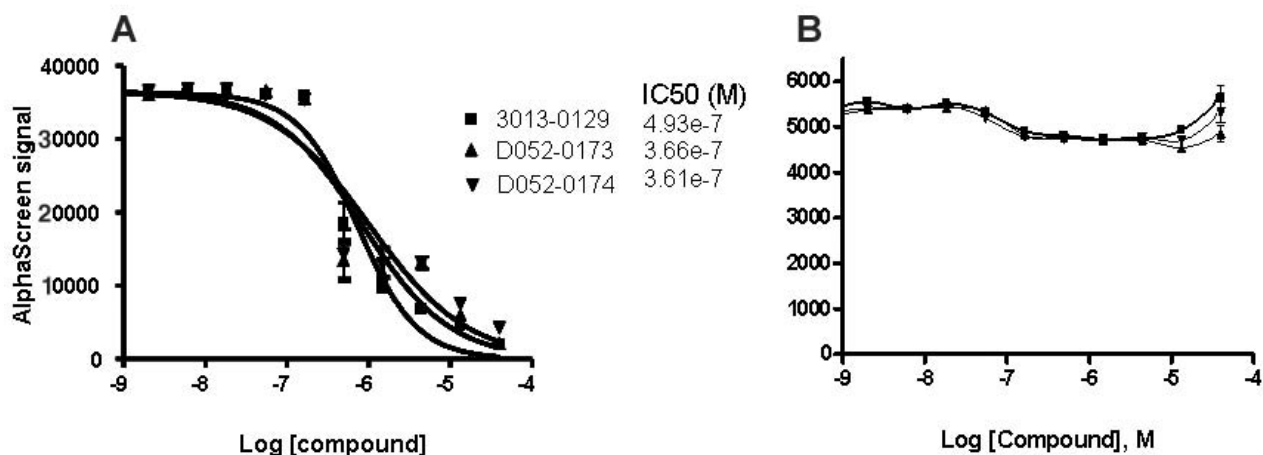


FIG. 6. AlphaScreenTM-detected (A) competition curves and (B) counterscreen of 3 hit compounds from the 76,314-compound NIH Chemical Genomics Center (NCGC) library.

assay buffer to minimize promiscuous inhibition that could be caused by compound aggregation.¹⁸ We selected the active compounds based on their potencies instead of the percentage inhibition in the traditional single-concentration screening. A selection criterion of IC₅₀ values <10 μM and maximal activity >50% was used to identify 149 active compounds as the primary “hits” from this screen.

Identifying target-independent positives using the TrueHits screen or a counterscreen

In the compound screen process, each assay format may produce its assay-specific false positives in addition to the screen operation-related target-independent positives such as lint and dispensing glitch. In this assay design, the Ni²⁺ chelate-coated beads were used to capture the TPR2A-Histag protein, which could interfere with the metal chelators in the compound library. The singlet oxygen transferring between the Acceptor and Donor beads might also be interrupted by certain compounds in the compound collection. In addition, the binding of the biotin-Hsp90 peptide and streptavidin-coated beads could be blocked by the compounds such as the biotin analogs.

To identify target-independent positives, we applied the AlphaScreenTM TrueHits kit to screen the 60 “hit” compounds identified from the 20,000 Maybridge compounds. Interfering compounds were identified as those with >30% inhibition. No interfering compound was identified from the 60 “hits” from the 20,000 compounds. We then measured the full competition curves for the 60 compounds. Target-independent positives typically exhibit nonsigmoidal or even linear competition curves. Thirty-one of the 60 “hit” compounds displayed “normal” dose-dependent and sigmoidal responses (see Supplementary Figure S1 at <http://jbx.sagepub.com/supplemental>). All compounds had

IC₅₀ values on average similar to that of the free Hsp90 C-terminal peptide. Their Hill slopes were close to -1, suggesting there was no cooperativity in the competitive compounds binding to TPR2A and no aggregation of reagents in the assay.¹⁹

From the NCGC screen, 149 “primary hits” were identified based on their <10 μM IC₅₀ values (see PubChem AID = 595). Forty-one compounds representative of different structural clusters were selected, reordered, and retested. The binding of 39 of these 41 compounds was confirmed. These 39 compounds were tested in the counterscreen assay (see PubChem AID = 632). Thirty-six of these 39 were positive in the counterscreen assay with sigmoidal shape competition curves and thus were discarded as interfering compounds. Among these, some had imidazole-like structures, implying that they were competing with the His₆-tagged protein for binding to the nickel-chelated Acceptor beads. Many other mechanisms of nonspecific inhibition are possible, such as being singlet oxygen or fluorescence quenchers.

The final 3 confirmed NCGC compounds had close activity in the competition assay, with IC₅₀ values of 0.49, 0.37, and 0.36 μM, respectively (Fig. 6), likely due to their highly related structures. It is worth noting that even though this class of compounds was not active in the counterscreen, they must be further characterized and confirmed using independent assays. We do not expect the counterscreen to be 100% effective in ruling out all target-independent positives, but at least they allow us to trim the “hit” list and remove compounds that are certainly *not* true positives.

CONCLUSION

We have established the first HTS assay to identify compounds that inhibit the Hsp90-HOP interaction using

AlphaScreen™ technology. The assay can distinguish between ligands with different affinities for TPR2A, as with free Hsp90 and Hsp70 peptides. The average Z' factor value was over 0.7, indicating that the assay can adapt well to a high-throughput assay format. By screening this assay against a total of 96,174 compounds from different sources, we identified 209 primary “hits” that potentially interrupt the Hsp90-HOP interaction in vitro. After excluding target-independent positives using a TrueHits kit screen or a counterscreen, followed by full concentration-response curve measurements, we have 34 “hit” compounds (overall 0.03% hit rate) with average IC_{50} values in the micromolar range (see Supplementary Figure S2 at <http://jbx.sagepub.com/supplemental>). Further characterization of these “hits” using secondary in vitro and in vivo assays will be presented elsewhere.²⁰

This study represents the first effort to identify a novel class of Hsp90 inhibitors by interrupting its cochaperone interaction. The compounds identified using this assay have the potential to be used as molecular probes to study the role of cochaperones in Hsp90-dependent cellular function and signaling pathways. Although a major attraction of current Hsp90 inhibitors is their ability to simultaneously inhibit multiple signaling pathways, the pleiotropic effects resulting from inhibition of Hsp90 ATPase activity also make it very difficult to determine which specific Hsp90 client proteins or signaling pathways are the most critical for the anticancer activity of Hsp90 inhibitors in a particular tumor. Because the specificity of Hsp90 for different client proteins is to some extent determined by its cochaperones,^{21,22} it is reasonable to speculate that inhibitors that inhibit Hsp90 function by disrupting its specific cochaperone interaction might provide the attractive feature of specific inhibition of client proteins.

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REFERENCES

1. Neckers L: Heat shock protein 90: the cancer chaperone. *J Biosci* 2007;32:517-530.

2. Powers MP, Workman P: Inhibitors of the heat shock response: biology and pharmacology. *FEBS Lett* 2007;581:3758-3769.
3. Chiosis G, Timaul MN, Lucas B, Munster PN, Zheng, FF, Sepp-Lorenzino L, et al: A small molecule designed to bind to the adenine nucleotide pocket of Hsp90 causes Her2 degradation and the growth arrest and differentiation of breast cancer cells. *Chem Biol* 2001;8:289-299.
4. Hardcastle A, Tomlin P, Norris C, Richards J, Cordwell M, Boxall K, et al: A duplexed phenotypic screen for the simultaneous detection of inhibitors of the molecular chaperone heat shock protein 90 and modulators of cellular acetylation. *Mol Cancer Ther* 2001;6:1112-1122.
5. Goetz MP, Toft D, Reid J, Ames M, Stensgard B, Safgren S, et al: Phase I trial of 17-allylamino-17-demethoxygeldanamycin in patients with advanced cancer. *J Clin Oncol* 2005;23:1076-1087.
6. Solit DB, Ivy SP, Kopil C, Sikorski R, Morris MJ, Slovin SF, et al: Phase I trial of 17-allylamino-17-demethoxygeldanamycin in patients with advanced cancer. *Clin Cancer Res* 2007;13:1775-1782.
7. Ramanathan RK, Egorin MJ, Eiseman JL, Ramalingam S, Friedland D, Agarwala SS, et al: Phase I and pharmacodynamic study of 17-(allylamino)-17-demethoxygeldanamycin in adult patients with refractory advanced cancers. *Clin Cancer Res* 2007;13:1769-1774.
8. Caplan AJ: What is a cochaperone? *Cell Stress Chaperones* 2003;8:105-107.
9. Pearl LH, Prodromou C, Workman P: The Hsp90 molecular chaperone: an open and shut case for treatment. *Biochem J* 2008;3:439-453.
10. Chen S, Smith DF: Hop as an adaptor in the heat shock protein 70 (Hsp70) and hsp90 chaperone machinery. *J Biol Chem* 1998;273:35194-35200.
11. Scheufler C, Brinker A, Bourenkov G, Bartunik H, Hartl FU, Moarefi I: Structure of TPR domain-peptide complexes: critical elements of the assembly of the Hsp70-Hsp90 multichaperone machine. *Cell* 2000;101:199-210.
12. Brinker A, Scheufler C, von der Mulbe F, Fleckenstein B, Herrmann C, Jung G, et al: Ligand discrimination by TPR domains: relevance and selectivity of EEVD-recognition in Hsp90-HOP-Hsp90 complexes. *J Biol Chem* 2002;277:19265-19275.
13. Chen MS, Silverstein AM, Pratt WB, Chinkers M: The tetratricopeptide repeat domain of protein phosphatase 5 mediates binding to glucocorticoid receptor heterocomplexes and acts as a dominant negative mutant. *J Biol Chem* 1996;271:32315-32320.
14. Cortajarena AL, Yi F, Regan L: Designed TPR modules as novel anticancer agents. *ACS Chem Biol* 2008;3:161-166.
15. Ullman EF, Kirakossian H, Singh S, Wu ZP, Irvin BR, Pease PS, et al: Luminescent oxygen channeling immunoassay: measurement of particle binding kinetics by chemiluminescence. *Proc Natl Acad Sci USA* 1994;91:5426-5430.
16. Warner G, Illy C, Pedro L, Roby P, Bosse R: AlphaScreen kinase HTS platforms. *Curr Med Chem* 2004;11:721-730.
17. Inglese J, Auld DS, Jadhav A, Johnson RL, Simeonov A, Yasgar A, et al: Quantitative high-throughput screening: a titration-based approach that efficiently identifies biological activities in large chemical libraries. *Proc Natl Acad Sci USA* 2006;103:11473-11478.
18. Feng BY, Shoichet BK: A detergent-based assay for the detection of promiscuous inhibitors. *Nat Protocols* 2002;1:550-553.
19. McGovern SL, Helfand BT, Feng B, Shoichet BK: A specific mechanism of nonspecific inhibition. *J Med Chem* 2003;46:4265-4272.
20. Yi F, Regan L: A novel class of Hsp90 inhibitors. *ACS Chem Biol*. In press.

21. Roe SM, Ali MM, Meyer P, Vaughan CK, Panaretou B, Piper PW, et al: The mechanism of Hsp90 regulation by the protein kinase-specific cochaperone p50^{cdc37}. *Cell* 2004;116:87-98.
22. Riggs DL, Cox MB, Cheung-Flynn J, Prapapanich V, Carrigan PE, Smith DF: Functional specificity of cochaperone interactions with Hsp90 client proteins. *Crit Rev Biochem Mol Biol* 2004;39:279-295.

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